MMAS-1, the Branch Point Between cis- and trans-Cyclopropane-containing Oxygenated Mycolates in Mycobacterium tuberculosis*

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Ying Yuan‡, Deborah C. Crane‡, James M. Mussers‡, Srinand Sreevatsans§, and Clifton E. Barry, III†‡¶

From the ‡Tuberculosis Research Unit, Laboratory of Intracellular Parasites, Rocky Mountain Laboratories, NIAID, National Institutes of Health, Hamilton, Montana 59840 and the §Section of Molecular Pathobiology, Department of Pathology, Baylor College of Medicine, Houston, Texas 77030

The proportion of mycolic acid containing trans-substituents at the proximal position of the meromycolate chain is an important determinant of fluidity of the mycobacterial cell wall and is directly related to the sensitivity of mycobacterial species to hydrophobic antibiotics. MMAS-1, an enzyme encoded in the gene cluster responsible for the biosynthesis of methoxymycolates, was overexpressed in Mycobacterium tuberculosis and shown to result in the overproduction of trans-cyclopropane and trans-olefin-containing oxygenated mycolic acids. MMAS-1 converted a cis-olefin into a trans-olefin with concomitant introduction of an allylic methyl branch in a precursor to both the methoxy and ketone-containing mycolic acids. In addition to an increase in the amount of trans-mycolate, MMAS-1 expression resulted in a substantial increase in the amount of keto-mycolate produced relative to methoxymycolate. Thus MMAS-1 may act at a complex branch point where expression of this enzyme directly affects the cis-to trans-ratio and indirectly affects the keto to methoxy ratio. Overexpression of MMAS-1 resulted in a substantially slower growth rate at moderately elevated temperature, decreased thermal stability of the cell wall as measured by differential scanning calorimetry, and an increased permeability to chenodeoxycholate. These results provide experimental evidence for the intermediacy of trans-olefinic mycolate precursors in trans-cyclopropane formation and suggest that increasing the proportion of the polar ketomycolate subclass may exert a significant fluidizing effect on the cell wall.

*Mycobacterium tuberculosis* is an important pathogen of man that displays an intrinsic resistance to many standard antimicrobials (1, 2). An important factor in this resistance is the formidable permeability barrier imposed by the mycobacterial cell wall (3–8). This barrier is formed by a lipid-rich complex heteropolymer composed of covalently linked subunits of pentadiglycerol, arabinogalactan, and mycolic acids (3). The barrier function of the cell wall to entry of hydrophobic solutes is due to a parallel alignment of mycolic acids in the inner leaflet of the cell wall (9, 10).

Mycolic acids are very long chain two-branched, three-hydroxy fatty acids that range from 75 to 88 carbons in total length in the tubercle bacilli (3, 11–13). Several unique structural features of these molecules may play a direct role in allowing the formation and determining the properties of the asymmetrical lipid bilayer, which is characteristic of mycobacteria and closely related genera (4). The primary division of mycolic acids into subclasses is dependent upon the presence or the absence of oxygen-containing functional groups in the longer (mero) chain (11). The proximal position (nearer the β-hydroxy acid) contains exclusively cis- or trans-olefin or cyclopropane, whereas the distal position may contain the same or one of a variety of oxygen moieties such as α-methyl ketone, α-methyl methyl ether, methyl-branched ester, or an α-methyl epoxide. The exact subclasses of mycolates present, and their relative quantities are unique to an individual species or very closely related species under defined growth conditions (14–16). In *M. tuberculosis* there are three classes of mycolic acids produced (see Fig. 1) (17–19). In the α series the meromycolate chain bears two cis-cyclopropanes. In the methoxymycolate series the meromycolate chain bears a methoxy group with an α-methyl branch in the distal position and a cis- or α-methyl trans-cyclopropane at the proximal position. In the ketomycolate series the meromycolate chain bears a ketone with an α-methyl group and a cis- or α-methyl trans-cyclopropane in the proximal position (18). In the tubercle bacilli the shorter α-mycolates are generally predominant followed closely by methoxymycolates with significantly less ketomycolates (14, 20).

α-Mycolates, containing only olefins or cyclopropanes in the mero chain, are cyclopropanated as protection against oxidative damage in pathogenic species of mycobacteria (21). cis-Cyclopropanation in the proximal mycolate position has a small negative effect on the fluidity of the mycobacterial cell wall but increased trans-cyclopropanation of this same position appears to be directly and much more strongly correlated with decreased fluidity of the resulting membrane system (22, 23). Oxygenated and α-mycolate species frequently contain a mixture of both cis- and trans-cyclopropane (or olefin) isomers depending upon the species (*M. tuberculosis* only contains trans-cyclopropanes in the two oxygenated series) (11). The ratio of cis- to trans-mycolate in the cell wall is linearly related to growth temperature in some species such as *Mycobacterium avium* and *Mycobacterium smegmatis* with increasing temperatures resulting in increasing amounts of trans-mycolate present in the cell wall (23). Thus trans-mycolates represent an important modification system allowing mycobacteria to maintain constant membrane viscosity in the face of changing environmental conditions. Oxygenated mycolate species have been proposed to play a role in allowing the interaction of more loosely associated cell wall molecules by providing hydrogen bonding opportunities within this lipid-rich environment (11).
Ketomycolate species have been shown to be more abundant during exponential growth of Mycobacterium microti and to be less abundant relative to methoxymycolate during stationary phase (16). In addition, ketomycolates were abundant in cell walls of M. microti harvested from mouse lungs (16).

The enzymes responsible for the biosynthesis of both cis-cyclopropanes in the α series of mycolates in M. tuberculosis have been identified as have the enzymes responsible for the biosynthesis of the methoxy, methyl, and cis-cyclopropane of methoxy mycolate (21, 22, 24). These five enzymes form a closely related family of methyl transferases that appear to share a common chemical mechanism beginning with methyl transfer to a cis-olefin (24). The fate of the cation formed by this methylation determines the structure of mycolic acid formed. In the case of CAMS-1, CAMS-2, and MMAS-2, proton abstraction from the incoming methyl group results in the formation of a cis-cyclopropane. In the case of MMAS-4, the addition of water to the intermediate cation results in hydroxymycolate formation and MMAS-3 functions to transfer a methyl group to the hydroxymycolate formed by MMAS-4. The genes encoding MMAS-2, -3, and -4 are clustered with a gene encoding a sixth putative methyl transferase designated MMAS-1 whose expression had no effect on the mycolates produced in M. smegmatis (24). The present study was undertaken to understand the function of the MMAS-1 enzyme and its potential role in trans-methoxymycolate formation.

EXPERIMENTAL PROCEDURES

Growth Conditions and Strains—M. tuberculosis strain H37Rv (ATCC 27294) was grown in roller bottles in Middlebrook 7H9 medium with albumin-dextrase-catalase supplement (Remel, Lenexa, Kansas) and Tween 80 (0.05%) (CalBiochem) containing, where appropriate, kanamycin (25 μg/ml) (Sigma). Recent clinical isolates of M. tuberculosis were grown on Lowenstein-Jensen slants (Remel, Lenexa, Kansas) and used to inoculate liquid cultures as above. All M. tuberculosis isolates were grown in the BL-3 containment facility at the Rocky Mountain Laboratories. Growth of strains was monitored by removing aliquots and measuring the A_{680 nm} daily. Chenedoxycholate uptake studies were performed as described previously (23). These organisms were recovered in 1994 or 1995 from patients with tuberculosis in Houston, TX. The bacteria have been passaged fewer than three times on Lowenstein-Jensen medium and represent ten distinct IS6110 subtypes and the three main genetic groups into which all M. tuberculosis isolates can be assigned based on the combination of sequence polymorphisms located at codon 463 of the catalase-peroxidase gene (katG) and codon 95 of the gene encoding the A subunit of DNA gyrase (gyrA) (26). The isolates were all susceptible to isoniazid, rifampin, streptomycin, and ethambutol.

Recombinant DNA Constructs and Techniques—M. tuberculosis was transformed with various constructs as described previously (27, 28). Briefly, we collected 200 ml of an actively growing culture with an A_{680 nm} = 0.5 by centrifugation at 5000 × g for 10 min and resuspended in 200 ml of cold distilled water. The bacteria were collected by centrifugation and resuspended a second time. Following a third centrifugation the bacteria were resuspended in 50 ml of cold distilled water and pelleted one final time. The pellet was resuspended in 5 ml of distilled water, and 0.4-ml aliquots were mixed with <10 μl DNA in a low salt buffer. Cells were electroporated in 0.2-cm cuvettes at 2.5 kV, 1000 Ohms, 25 microfarads using a commercial electroporation apparatus (Bio-Rad) and immediately transferred into 5 ml of 7H9, albumin-dextrase-catalase supplement, Tween without antibiotics. The cells were recovered at 37 °C for 24 h before plating onto Middlebrook 7H11 medium (Difco) containing kanamycin.

pMV206 was provided by MedImmune, Inc. (Gaithersburg, MD). The mma1 subclone has been previously described (24). The sequence upstream of mma1 was determined by polymerase chain reaction amplification of the 500 nucleotides upstream of mma1 from H37Rv, Mycobacterium bovis BCG, HN35, and HN40 using the following two primers, 5’-CCCGGATCCTAGGCGCAGGTGTAGACC-3’ and 5’-CGCGGATCCTAGGCGCAGGTGTAGACC-3’. The polymerase chain reaction product was cloned into pBluescript KS+, and the nucleotide sequence was determined on both strands using universal primers.

Mycolic Acid Isolation, Purification, and Analysis—Methylocyclooctane was prepared and purified as described previously (21, 29). Analytical one-dimensional and two-dimensional TLC were performed as described previously (22). Individual mycolate classes were isolated by preparative TLC on 1000 μm of Silica Gel 60 using five developing systems with 0.5:9.5 ethyl acetate:hexanes followed by visualization by spraying with 0.01% Rhodamine 6G (Sigma) in ethanol. Methylocyclooctane were eluted three times with diethyl ether and the eluant filtered through 0.45 μm of polyvinylidene difluoride filters before removing the ether and reprecipitating (29). For electrospray mass spectrometry, mycolates purified to class as methyl esters were resaponified overnight by treatment with 15% potassium hydroxide in aqueous methanol (50%) at 70 °C. Acidification and extraction with ether was followed by washing with distilled water and drying under reduced pressure. The residue was dissolved in toluene:acetonitrile and reprecipitated for analysis.

Analytical Techniques—500-MHz proton NMR spectra were recorded in deuterochloroform on a Bruker AM500 NMR spectrometer (NuMeGa Resonance Labs, San Diego, CA). Electrospray mass spectrometry was performed on a API III Perkin-Elmer SCIEX mass spectrometer (Mass Spectrometry Consortium, San Diego, CA) using dichloromethane:chloroform:methanol (1:1:1) as solvent. Samples were introduced at 4 μl/min through a 100-μm orifice with a declustering potential of between 50 and 200V. Differential scanning calorimetry (DSC) was performed using a model 4110 DSC (Calorimetry Sciences Corp., Provo, UT) as described previously (22).

RESULTS

Overexpression of Mma1 in M. tuberculosis—The lack of a phenotype upon transformation of M. smegmatis with plasmids bearing mma1 suggested that either the protein was not efficiently produced in M. smegmatis or that the substrate for the enzyme was not produced in this heterologous organism (24). The appearance in Coomassie Brilliant Blue-stained SDS-polyacrylamide gels of a band of the appropriate molecular weight in transformants carrying only the mma1 gene appeared to rule out the first possibility. The production of the cis-isomer of the methoxymycolate series in M. smegmatis argued strongly that the substrate for the trans-isomer would be present because these two species are biosynthetically related (Fig. 1) (11). A third potential interpretation of these results was that the mma1 gene product was functional in M. smegmatis but did not result in the formation of a new mycolate species. This might be the case if, for example, the activity of MMAS-1 involved the equivalent of the interconversion of the α1 and α2 subclasses of mycolates in M. smegmatis. Because M. smegmatis is capable of regulating the ratio of α1 and α2 in response to physical changes in environment (23) it seemed possible that overexpression of the enzyme that produced α2 might result in the down-regulation of the normally expressed enzyme, which serves the same function resulting in a wild type mycolate profile.

To test this proposal we transformed wild type M. tuberculosis H37Rv with the plasmid pMV206 carrying a 1.2-kilobase NaeI to BamHI fragment containing only the mma1 open reading frame previously described (24). Kanamycin-resistant transformants were selected and grown in the presence of [14C]acetate. Mycolic acid methyl esters were purified from these cultures as described under “Experimental Procedures.” Transformants carrying mma1 appeared more slowly on plates and formed smaller, upright, more projectile drug colonies than control organisms transformed with only pMV206 without an insert. Two-dimensional TLC analysis of mycolates prepared from such colonies revealed a change in mycolate patterns as shown in Fig. 2. Fig. 2A shows a typical wild type M. tuberculosis pattern of α-mycolate (α), methoxymycolate (M), and ketomycolate (K). The primary separation observed represents the difference in polarity of these mycolate species, the

1 The ten clinical isolates studied are from the collection of J. M. M.

2 The abbreviation used is: DSC, differential scanning calorimetry.
second dimension, impregnated with silver ions, separates based upon number of double bonds. Wild type mycolates have no olefinic resonances and cis- and trans-cyclopropanes are not distinguished in this system. The pattern in Fig. 2B showed that mma1-transformed M. tuberculosis had the same three mycolates but also contained two additional components that migrated with identical polarity to methoxymycolate (1) and ketomycolate (2). These were retarded in the presence of silver ions, suggesting the presence of olefinic mycolates.

To more precisely define the effect of MMAS-1 expression on M. tuberculosis, total mycolates were isolated from both the recombinant and control organism and analyzed by 500 MHz proton NMR (Fig. 3). Methyl mycolates from control organisms showed nearly exclusively cis-cyclopropane resonances at δ = 0.87, 0.92 ppm and no downfield resonances associated with the presence of olefinic protons (Fig. 3A). The relative amount of methoxymycolate and ketomycolate in such samples could be assessed easily by comparing methyl branch doublet intensity in the region near the terminal methyl group triplet (50.85 ppm for methoxy, 51.05 ppm for keto). The ratio of keto to methoxy in this control sample was thus 1:3, respec-

![Diagram](Image)  
**Fig. 1. Mycolate class distribution in M. tuberculosis.** α-Mycolates are the most abundant mycolic acid type and contain no oxygen functionalities outside of the β-hydroxy acid moiety. The longer (mero) chain contains two cis-cyclopropanes. Methoxymycolates contain an α-methyl methyl ether moiety in the distal position and a cis-cyclopropane (Mc) or an α-methyl trans-cyclopropane (Mt) in the proximal position. Methoxymycolates are the second most abundant class of mycolic acid in M. tuberculosis. The much less abundant ketomycolates contain an α-methyl ketone moiety in the distal position and proximal functionalities as in the methoxy series (Kc and Kt).

![Diagram](Image)  
**Fig. 2. Two-dimensional TLC analysis of purified mycolic acids from wild type M. tuberculosis (carrying a control plasmid pMV206, A) and M. tuberculosis H37Rv (carrying pMV206:mma1, B).** α-mycolates (α), methoxymycolates (M), and ketomycolates (K) are marked, and the structures have been confirmed by 1H NMR analysis of each class following purification by preparative TLC. Compounds 1 and 2 are methoxymycolate and ketomycolate containing trans-olefin as described in the text. This TLC system has been described previously and consists of a normal phase silica gel separation in the right to left dimension and an argentation silica separation in the bottom to top dimension.

In agreement with the two-dimensional TLC data in Fig. 2B, the 1H NMR spectrum of total mycolates from the MMAS-1-expressing sample showed several major differences (Fig. 3B). First this sample showed resonances centered at 85.33 ppm clearly associated with olefinic protons with splitting patterns very similar to that observed in the α2 series of M. smegmatis (24). Interestingly, along with the appearance of an allylic methyl group doublet at 80.92 ppm and a measured coupling constant of J = 15Hz for the olefinic protons strongly suggests the presence of mycolate species containing a trans-olefin with adjacent methyl branch. The second important feature of this spectrum was the appearance of signals associated with a trans-cyclopropane at 80.15 and 0.47 ppm. The methyl group adjacent to the trans-cyclopropane occurs as a poorly resolved doublet slightly upfield of the terminal methyl triplet at 80.87 ppm. It should also be noted that the olefinic resonances represented two protons, whereas the trans-cyclopropane resonances accounted for four protons, thus the quantity of trans-olefin in these spectra is underestimated by a factor of two when directly comparing the integration areas. An additional feature of this spectrum worth noting was the increase in the amount of ketomycolate present relative to methoxymycolate.

**Effect of MMAS-1 on trans-Mycolates in Individual Mycolate Subclasses—** To quantitate the changes in relative amounts of mycolates present by subclass in MMAS-1 expressing M. tuberculosis, we examined two-dimensional TLC patterns of 14C-labeled methyl mycolates isolated from control and mma1-transformed organisms by phosphorimaging (Fig. 4). In control organisms α-mycolates represented about 51% of the total, methoxymycolates represented about 36%, and ketomycolates represented about 13%. In mma1-transformed organisms α-mycolates were slightly increased to 59% of the total, whereas methoxymycolates were significantly decreased in quantity to 12%, and ketomycolates were increased in relative abundance to 29% of the total.

In addition to a shift in the distribution of mycolate subclasses there was also a distinct bias in trans-mycolate distribution observed in the 1H NMR spectra of the various purified mycolate classes. α-Mycolate spectra were identical in control and mma1-transformed M. tuberculosis and contained only cis-cyclopropanes. In contrast, methoxymycolates contained less than 5% trans-cyclopropane and no trans-olefin in control mycolates.
Biosynthesis of trans-Mycolic Acids

samples, but in mma1-expressing organisms 60% of methoxymycolates contained trans-olefin or cyclopropane (Table I). Ketomycolates were affected even more dramatically. Whereas control samples showed about 20% trans-cyclopropane (and no trans-olefin), mma1-transformed organisms were 100% trans-olefin and cyclopropane.

Mycolic acids occur naturally as a series of related compounds differing in molecular weight by 28 atomic mass units (two methylene units) (30). The introduction of a methyl branch in the trans-cyclopropane series would be expected to increase the trans-geometry that grew significantly more slowly than control vectors. MMAS-1-expressing organisms (shifted up by 14 atomic mass units).

Table I

| Series | Total | trans-Olefin | trans-Cyclopropane |
|--------|-------|--------------|-------------------|
| WT     | α     | 0            | 0                 |
|        | M     | <5           | <5                |
|        | K     | 20           | 20                |
| MMAS-1 | α     | 0            | 0                 |
|        | M     | 60           | 40                |
|        | K     | 100          | 70                |

1) with the major isomers occurring at 1252 and 1280 atomic mass units. Upon introduction of mma1 the series of isomeric peaks in the mass spectrum appeared more complex, with major peaks at 1266, 1280, and 1294, the masses expected for the α-methyl, trans-cyclopropyl methoxymycolate, and the α-methyl trans-olefinic methoxymycolate (methoxymycolate with only an α-methyl trans-olefin has the same molecular mass as α-methylmethoxymycolate with a cis-cyclopropane). The mero chain in the ketomycolate series in wild type organisms had an identical number of carbons as in methoxymycolate but occurs at 14 atomic mass units lower molecular weight due to the absence of the methyl ether and the presence of a ketone in place of an alcohol. Thus the major isomers of ketomycolates were at 1236 and 1264 atomic mass units in wild type and 1250 and 1278 atomic mass units in MMAS-1-expressing organisms (shifted up by 14 atomic mass units).

Effect of MMAS-1 Expression on Growth Rate—M. tuberculosis expressing MMAS-1 formed colonies with unusual morphology that grew significantly more slowly than control vector-containing organisms on solid media. Transformations with mma1 often yielded relatively few colonies and many colonies displayed intermediate morphology. Loss of altered colony morphology was associated with loss of the trans-mycolate phenotype and the slow growth on plates selected for organisms that

FIG. 3. 500 MHz proton NMR spectra of total methyl mycolates purified from wild type M. tuberculosis (carrying a control plasmid pMV206, A) and M. tuberculosis H37Rv (carrying pMV206:mma1, B). The arrow in B at 5.25 ppm highlights the trans-olefinic protons which have a measured coupling constant of J = 15 Hz confirming the trans-geometry. The arrow at 8.15 ppm highlights the trans-cyclopropane resonances in the MMAS-1-expressing strain.

FIG. 4. Mycolate class distribution in wild type M. tuberculosis (carrying a control plasmid pMV206, 1) and M. tuberculosis H37Rv (carrying pMV206:mma1, 2). Methylycolates were prepared from samples radiolabeled with [14C]acetic acid, and the total amount of each class was determined by PhosphorImager analysis of two-dimensional TLC plates. White bars are α-mycoclates, gray bars are methoxymycolates, and black bars are ketomycolates.
had lost this colony morphology. However, growth of single-colony isolated transformants in liquid media at 37 °C revealed only a moderately slower growth rate when compared with control organisms (Fig. 5A). MMAS-1-expressing organisms did show a somewhat extended lag phase before reaching logaritmic growth. *M. tuberculosis* is a strict mesophile with only a narrow temperature range permissive for growth (33). Cultures fail to grow at 45 °C and are modestly inhibited by growth at 42 °C. To increase the stress on the organism due to changes in cell wall composition, we subcultured control and MMAS-1-expressing organisms identically in liquid culture at 42 °C and observed a dramatic decline in the growth rate of the recombinant MMAS-1 expressors with a much more modest effect on the growth rate of vector bearing controls (Fig. 5B).

**Mycolate Structure and Subclass in Laboratory Strains and Recent Clinical Isolates**—In the original description of the structure of the methoxy and ketomycolate series from *M. tuberculosis*, the methoxy series was found to be about 10% trans-cyclopropane, whereas the ketomycolate series was found to be 67% trans-cyclopropane (18). The amount of trans-cyclopropane observed in our H37Rv was representative of the wild type condition. In other mycobacterial species we have previously observed that the trans-percentage is reflective of certain growth conditions (23). We explored a number of different solid and liquid media including complex solid media such as Lowenstein-Jensen slants and simplified salts media such as glucose-alanine salts and Sauton’s medium with [**trans**]-cyclopropane much more rapidly than did control organisms. We then examined the mycolate composition of a series of 10 recent clinical isolates with a much more limited passage history than H37Rv (Table III). These isolates represent the breadth of species diversity found in *M. tuberculosis* strict sense and encompass 10 distinct IS 6110 subtypes. Quantitation of the mycolate classes from two-dimensional TLC analysis revealed relatively little variation in the amount of the three major classes. On average α-myculates comprise 57%, methoxy-myculates comprise 33%, and ketomycolates comprise 11% of the total mycolates present in each strain. Although there was no significant difference in the relative amounts of each class of mycolates, there was a difference in the amount of trans-cyclopropane present in H37Rv and in the related strain H37Ra, both of which had five times less trans-cyclopropane compared with the 10 clinical isolates. The introduction of MMAS-1 into H37Rv results in a strain with both altered mycolate classes and 2-fold higher trans-cyclopropane content than the clinical isolates.

These results suggest that the H37R strains may have been laboratory-selected for lower trans-cyclopropylmycolate content with a higher permeability cell wall and faster growth rate. We examined the region upstream of the *mma1* gene for promoter mutations by polymerase chain reaction amplifying the upstream 500 nucleotides (contained in GenBank accession number U66108) from H37Rv and two clinical isolates (HN35 and HN40). DNA sequence analysis revealed no alterations in this region in the laboratory strain (data not shown).

**Effect of Increased trans-Mycolate on Cell Wall Thermochemistry and Drug Uptake**—To explore more carefully the relationship between the altered mycolate structures and growth rate phenotype in MMAS-1-expressing H37Rv *in vitro*, we examined the stability of the cell wall complex by DSC of intact organisms (9, 22, 23). In control organisms, carrying vector and **K**-mycolates comprised 57%, methoxy-mycolates comprised 33%, and ketomycolates comprised 11% of the total mycolates present in each strain. Although there was no significant difference in the relative amounts of each class of mycolates, there was a difference in the amount of trans-cyclopropane present in H37Rv and in the related strain H37Ra, both of which had five times less trans-cyclopropane compared with the 10 clinical isolates. The introduction of MMAS-1 into H37Rv results in a strain with both altered mycolate classes and 2-fold higher trans-cyclopropane content than the clinical isolates.

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The lowered thermal transition temperature suggests a more fluid cell wall, which may allow for improved penetration of hydrophobic materials that cross the cell wall by diffusion through this barrier. To explore this possibility we measured the uptake of radiolabeled chenodeoxycholate in control and MMAS-1-expressing organisms (Fig. 7). Consistent with the DSC results, the MMAS-1-expressing organism took up chenodeoxycholate much more rapidly than did control organisms. This experiment was repeated several times with similar results. Uptake was also measured by growing organisms at 37 °C and then shifting to 42 °C prior to adding antibiotic. At 42 °C the same relative pattern of uptake was observed, with the MMAS-1-expressing strain taking up more antibiotic relative to control.

**Table II**

| Series | Molecular mass | 1108 | 1136 | 1164 | 1192 (m/e) |
|--------|---------------|------|------|------|------------|
| α      | WT            | 50   | 100  | 60   | 20         |
|        | MMAS-1        | 40   | 100  | 50   | 25         |
|        | **M**         | 1224 | 1252 | 1266 | 1280       |
|        | WT            | 65   | 100  | 10   | 10         |
|        | MMAS-1        | 20   | 50   | 25   | 10         |
|        | **K**         | 1236 | 1250 | 1264 | 1278       |
|        | WT            | 50   | 20   | 75   | 25         |
|        | MMAS-1        | 20   | 25   | 10   | 10         |

* Numbers refer to the relative intensity of the peak at the given mass value.

b M. methoxymycolates.

K. ketomycolates.

**Molecular mass**

| α      | 1224 | 1252 | 1266 | 1280 | 1294 | 1308 | 1324 (m/e) |
|--------|------|------|------|------|------|------|------------|
| **M**  | 65   | 100  | 10   | 80   | 10   | 20   | 5          |
|        | 20   | 50   | 25   | 45   | 40   | 25   | 25         |
| **K**  | 1236 | 1250 | 1264 | 1278 | 1292 | 1306 | 1306 (m/e) |
|        | 50   | 20   | 75   | 25   | 20   | 10   | 10         |
|        | 20   | 25   | 10   | 65   | 10   | 10   | 25         |
DISCUSSION

The potential functional roles of mycolic acids are dependent upon two basic properties; chain length and functional group (including oxygenated or nonoxygenated as well as configuration about olefinic or cyclopropyl groups). Some of the biosynthetic machinery responsible for functional group introduction in the tubercle bacillus has recently been identified. The two enzymes that introduce cis-cyclopropanes into the α-mycolate series have been identified as have three enzymes responsible for biosynthesis of methoxymycolates containing a cis-cyclopropane (21, 22, 24). These five enzymes form a family of S-adenosyl-L-methionine-dependent methyl transferases which transfer the methyl group of S-adenosyl-L-methionine to a lipid substrate.

Clustered with the three enzymes responsible for cis-cyclopropyl methoxymycolate biosynthesis was another homologous enzyme that had no apparent effect on the mycolates of M. smegmatis despite being actively expressed. Expression of this enzyme, MMAS-1, in M. tuberculosis, resulted in an increase in the amount of mycolate containing trans-cyclopropane and the appearance of mycolates containing trans-olefin with an allylic methyl branch. This effect was limited to the oxygenated types of mycolates that contain either an α-methyl methyl ether or an α-methyl ketone in the distal position in addition to the cis- or trans-cyclopropane (or olefin) in the proximal position. The increase in the amount of trans-cycloproyl mycolate, coupled with the presence of substantial amounts of trans-olefinic mycolate suggests that the function of MMAS-1 is the conversion of a cis-olefin containing oxygenated mycolate precursor into a trans-olefin with an adjacent methyl branch (Fig. 8). As we

Table III

| Strain      | TLC  | NMR (trans) | NMR (trans) |
|-------------|------|-------------|-------------|
| HN34        | 64   | 29          | 7           | 0.08         |
| HN35        | 62   | 29          | 9           | 0.11         |
| HN40        | 61   | 28          | 12          | 0.09         |
| HN59        | 54   | 36          | 10          | 0.10         |
| HN88        | 55   | 29          | 16          | 0.09         |
| HN93        | 59   | 31          | 9           | 0.08         |
| HN224       | 56   | 36          | 8           | 0.11         |
| HN386       | 66   | 26          | 8           | 0.07         |
| NN50        | 49   | 40          | 12          | 0.10         |
| HN50        | 55   | 34          | 11          | 0.12         |
| HN37Rv      | 51   | 36          | 13          | 0.02         |
| HN37Ra      | 51   | 36          | 13          | 0.03         |
| HN37Rv (mma1) | 59 | 12          | 29          | 0.20         |

* trans-Ratio for NMR is the (average signal intensity at 0.15 ppm)/(average signal intensity at 0.33 ppm).
have previously proposed, such an outcome is explainable for a member of this enzyme family by abstraction of a proton from the methylene carbon adjacent to the intermediate cation resulting from methyl group addition (24). This result suggests that the trans-olefinic mycolate with an allylic methyl branch is the biogenetic precursor to the trans-cyclopropane with an adjacent methyl branch, a proposal that has been made previously (11, 34).

MMAS-1 action appears to be entirely limited to the oxygenated types of mycolic acids. α-Mycolates from MMAS-1-expressing organisms have no trans-cyclopropane and no trans-olefin. This suggests that keto and methoxymycolates share a common biosynthetic precursor that occurs farther along the biosynthetic pathway than the point at which the oxygenated and α-mycolates diverge. The chain-length equivalence of the keto and methoxy mycolates (56 carbons in meromycolate chain of the major isomers) compared with the chain length of the α-mycolates (50 carbons in the meromycolate) suggests that these two biosynthetic branches may arise from distinct extension systems and may be only distantly related. The selectivity of MMAS-1 for the oxygenated branches supports this hypothesis. The surprising finding that the expression of MMAS-1 affects the relative levels of methoxymycolate and ketomycolate expressed by the cell suggests another more subtle connection between these two branches of the oxygenated mycolate pathway. One hypothesis that explains this result involves the specificity of the MMAS-3 enzyme relative to the oxidase that may transform the hydroxymycolate precursor into the keto-mycolate precursor (Fig. 8). This scheme proposes that MMAS-1 splits the pathway into two branches, a branch carrying cis-mycolate precursors and a branch carrying trans-mycolate precursors. MMAS-4 will accept either cis- or trans-olefin or cis-cyclopropane as a substrate (24), so the intermediate hydroxymethylmycolate precursor may exist as a mixture of cis- and trans-cyclopropane according to the relative expression level of MMAS-1. The class of mycolate (methoxy or keto) may be linked to the cis- or trans-geometry if either MMAS-3 or the unknown oxidase (or both) exhibit a preference for a substrate with a given proximal geometry. In this way the ultimate level of ketomycolate or methoxymycolate may be directly related to the action of MMAS-1. Alternatively ketomycolate overexpression may be a regulated response of the cell to declining fluidity caused by the increase in trans-mycolate species.

Mycolic acids are an integral part of the mycobacterial cell wall. The precise proportions of each class of mycolate and the structure of individual members of these classes has a potentially large impact on many putative functions of the cell wall.

**FIG. 7.** Uptake of 14C-labeled chenodeoxycholate by wild type *M. tuberculosis* (carrying a control plasmid pMV206, open circles) and *M. tuberculosis H37Rv* (carrying pMV206:mma1, closed circles). The results are reported as dpm of chenodeoxycholate taken up per mg of dry weight of cells.

**FIG. 8.** Biosynthetic pathway for cis- and trans-oxygenated mycolates and the function of the MMAS-1 gene product. The partial structures refer to a proposed meromycolate precursor whose precise structure is unknown. MMAS-1 serves to interconvert the cis- and trans-series as shown, and the functions of MMAS-2, MMAS-4 and MMAS-3 have been previously determined. The question marks refer to an as yet unidentified oxidase that is postulated to be capable of transforming the hydroxy mycolate precursor of either the cis- or trans-series into the corresponding ketomycolate precursor.
It has previously been demonstrated that the proportion of trans-mycolate is linearly related to the fluidity of the cell wall (23). In MMAS-1-overexpressing M. tuberculosis the amount of trans-mycolate (trans-olefin plus trans-cyclopropane) is about four times as high as occurs naturally. In addition the amount of ketomycolate is two to three times higher than normal. These alterations to cell wall structure have a profound effect on cell wall function and viability of the cell. Growth of the organism on solid media is impaired, surface morphology is distinctive, and growth in liquid media is not dramatically affected at normal growth temperatures; however, growth at slightly elevated temperature is severely impaired. DSC of the MMAS-1 overexpressor suggests that at least a portion of the defect is related to a change in permeability of the outer cell wall. The increase in fluidity signaled by the lowered DSC temperature was unexpected given the previous correlation of increasing trans-mycolate with increasing transition temperature. This result was consistent with measurement of uptake of the hydrophobic antibiotic chenodeoxycholate, which was increased in the MMAS-1 overexpressor.

There are two possible explanations for this phenomenon; first, there may be a threshold value for the amount of trans-mycolate below which the addition of trans-mycolate improves hydrocarbon chain packing and above which trans-mycolate interferes with tight packing. The very high ratios of trans-mycolate in M. smegmatis and M. avium suggest that this is not likely to be the case. The second explanation is that the increased amount of ketomycolate present in the cell wall of recombinant organisms has a fluidizing effect. This may reflect an increased association of hydrophilic molecules with the boundary region between the inner and outer leaflet of the cell wall due to an increase in the availability of hydrogen-bonding sites. A more extreme case of this was observed in M. smegmatis expressing only MMAS-4 in which hydroxymycolates were a major mycolate species produced (24). This organism was dramatically growth impaired and appeared to have the very wet surface morphology more characteristic of lipopolysaccharide containing Gram-negative organisms such as Escherichia coli. The higher relative abundance of ketomycolate over methoxy-mycolate during exponential growth of M. microti and the subsequent reversal of this ratio during stationary phase may be related to a general decline in fluidity after active growth (16). Combined, these observations suggest that the presence of polar ketone groups in the mycolate chain has an overall fluidizing effect on the cell wall, but this fluidizing effect is not directly coupled to growth rate, and more complex interactions in cell wall associated molecules may ultimately determine this property.

The occurrence and distribution of various mycolate subclasses in isolates of M. tuberculosis and related mycobacteria has received some attention (13, 24). In one examination of the laboratory strain H37Rα, α-mycolates were shown to comprise 77% of the total, methoxy-mycolates were shown to comprise 13%, and ketomycolates were shown to comprise 10% (35). Our results with H37Rα, H37Rv, and 10 recent clinical isolates of M. tuberculosis differ from these results in that the methoxy-mycolate proportion is significantly higher in all of these strains (Table III). Quantitation of the proportion of methoxy-mycolate by a comparison of the 1H NMR integration values for the methyl ester singlet at δ3.69 ppm and the methyl ether protons at δ3.34 ppm in total mycolate spectra appeared to confirm the quantitative data from phosphorimaging of 14C-labeled TLC plates (data not shown). A further examination of the literature concerning methoxy and ketomycolate subclasses revealed that the original isolation reported much higher values for the amount of trans-cyclopropane than we observed in current isolates of the H37Rv strain (18). Because of the observed effect on growth rate of transgenic H37Rv with mma1, we examined whether the decrease in observed trans-mycolate content was due to selection for organisms with a faster growth rate by extensive in vitro passage of such laboratory strains. In all of 10 recent clinical isolates of M. tuberculosis, the proportion of trans-mycolate was five times higher than in H37Rv. This result is much more consistent with the original description of the trans-mycolate content in the oxygenated subclasses by Minnikin and Polgar in 1967 (18, 19). The importance of trans-mycolate content to many macroscopic qualities of the tubercle bacilli (increased cell wall rigidity, increased drug resistance, potentially a decreased growth rate, and altered colony morphology, etc) has been previously established (23). The potential differences in these properties are significant enough that altered pathogenesis of laboratory strains that do not contain significant quantities of trans-oxygenated mycolates seems likely.

These results extend our earlier studies on the biosynthetic pathway for methoxymycolates and confirm the prediction that a member of the mycolate methyltransferase family of enzymes can function to introduce a trans-olefin with an α-methyl branch into a precursor mycolate (24). MMAS-1 offers a very attractive target for chemotherapeutic intervention due to the importance of trans-mycolates in cell wall structure and also due to the role of this activity in regulating the proportion of ketomycolate to methoxymycolate. Inhibiting MMAS-1 function in intact cells would be predicted to have the effect of inhibiting trans-mycolate formation and indirectly ketomycolate synthesis and thereby offers the potential for significant bacteriostatic effects. In addition, the ability to modify cell wall-bound mycolates may allow the creation of vaccine strains with altered mycolate profiles. The recent discovery that αβ T cells can specifically recognize and respond to mycolic acids presented by a CD1b cell surface glycoprotein suggests that modification of mycolate profiles in candidate vaccine strains may alter the host immune response to strains expressing a particular mixture of mycolates (36, 37). Finally the availability of six members of the mycolic acid methyltransferase family will facilitate the development of cell-free in vitro assays for methyl transfer and allow a more precise definition of the details of mycolic acid biosynthesis and modification.

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Ying Yuan, Deborah C. Crane, James M. Musser, Srinand Sreevatsan and Clifton E. Barry III

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