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The *Mycobacterium tuberculosis cmaA2* Gene Encodes a Mycolic Acid *trans*-Cyclopropane Synthetase*

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Infection with *Mycobacterium tuberculosis* remains a major global health emergency. Although detailed understanding of the molecular events of *M. tuberculosis* pathogenesis is still limited, recent genetic analyses have implicated specific lipids of the cell envelope as important effectors in *M. tuberculosis* pathogenesis. We have shown that *pcaA*, a novel member of a family of *M. tuberculosis S*-adenosyl methionine (SAM)-dependent methyl transferases, is required for α-mycolic acid cyclopropanation and lethal chronic persistent *M. tuberculosis* infection. To examine the apparent redundancy between *pcaA* and *cmaA2*, another cyclopropane synthetase of *M. tuberculosis* thought to be involved in α-mycolate synthesis, we have disrupted the *cmaA2* gene in virulent *M. tuberculosis* by specialized transduction. Inactivation of *cmaA2* causes accumulation of unsaturated derivatives of both the methoxy- and ketomycolates. Analysis by proton NMR indicates that the mycolic acids of the *cmaA2* mutant lack *trans*-cyclopropane rings but are otherwise intact with respect to cyclopropane and methyl branch content. Thus, *cmaA2* is required for the synthesis of the *trans* cyclopropane rings of both the methoxymycolates and ketomycolates. These results define *cmaA2* as a *trans*-cyclopropane synthetase and expand our knowledge of the substrate specificity of a large family of highly homologous mycolic acid methyl transferases recently shown to be critical to *M. tuberculosis* pathogenesis.

Mycobacterium tuberculosis infection continues to overwhelm the populations of the developing world. It has been estimated that in 1997 there were 8 million new cases of active tuberculosis that were added to the already existing 16 million cases (1). In the same year, 2 million people died of tuberculosis as a result of an astonishing case fatality rate of 23–50% (1). This high death rate for a disease treatable with available antibiotics reflects the geographic superimposition of HIV[1] and *M. tuberculosis* infection, and the logistical and economic burden of at least 6 months of multidrug therapy required to treat the disease. New drugs to shorten therapy and vaccine candidates to prevent *M. tuberculosis* infection are badly needed but will only come with a more thorough understanding of the mechanisms of *M. tuberculosis* pathogenesis.

The cell envelope of *M. tuberculosis* is a highly complex array of distinctive lipids and glycolipids that has been intensely scrutinized as a potential effector in the interaction of *M. tuberculosis* with the human host (2–4). Investigation into the role of the cell envelope in virulence has been hampered by a lack of defined mutants of *M. tuberculosis* that fail to synthesize specific components of the cell surface. Recently, advances in the genetic manipulation of *M. tuberculosis* have allowed isolation of several mutants with defined cell envelope deficiencies and altered virulence (5–7). *M. tuberculosis* synthesizes three classes of mycolic acids, very long chain α-alkyl, β-hydroxyl fatty acids (Fig. 1) in its cell envelope. These three classes of mycolic acids, α-, methoxy-, and ketomycolates, are modified with cyclopropane rings and methyl branches through the combined action of a large family of *S*-adenosyl methionine (SAM)-dependent methyl transferases that modify double bonds in the meromycolate chain. The oxygenated mycolic acids contain either *cis*- or *trans*-cyclopropane rings at their proximal position. Whereas the putative *cis*-cyclopropane synthetase of the methoxymycolates has been identified (8, 9), the *trans*-cyclopropane synthetase is unknown. *pcaA*, one of the members of this distinctive gene family, has been established as essential for *M. tuberculosis* pathogenesis because a mutant of *pcaA* cannot establish a chronic persistent *M. tuberculosis* infection in mice (6). Biochemically, *pcaA* is required for the synthesis of the proximal cyclopropane ring of the α-mycolate molecule (Fig. 1). The finding that *pcaA* was required for proximal cyclopropanation of the α-mycolate molecule was surprising because this function had been previously attributed to *cmaA2*, another cyclopropane synthetase of *M. tuberculosis* (3, 10, 11). When introduced into *Mycobacterium smegmatis* on a multicopy plasmid, *cmaA2* introduces *cis*-cyclopropane rings at the proximal position of the α-mycolate and the epoxymycolate, a position occupied by a double bond in the wild-type mycolates of this strain (11). Despite this lack of substrate specificity in *M. smegmatis*, the function of *cmaA2* in *M. tuberculosis* was thought to be proximal cyclopropanation of the α-mycolate molecule. Thus, the functions of *pcaA* and *cmaA2* appeared to overlap. To define the function of *cmaA2* and to more completely explore the substrate specificity of the SAM-dependent methyl transferases of *M. tuberculosis,* we have inactivated *cmaA2* in *M. tuberculosis* and shown here that *cmaA2* is the *trans*-cyclopropane synthetase for both the methoxy- and ketomycolates.

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[1] The abbreviations used are: HIV, human immunodeficiency virus; SAM, *S*-adenosyl methionine; bp, base pair(s); PCR, polymerase chain reaction.

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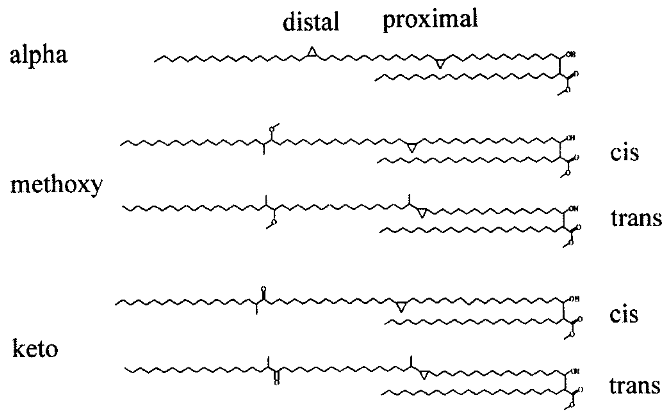

FIG. 1. **Structures of the mycolic acids of *M. tuberculosis*.** α-Mycolate contains two cyclopropane residues whereas methoxy- and keto-mycolate contain a proximal *cis*- or *trans*-cyclopropane ring and a distal oxygenated functional group.

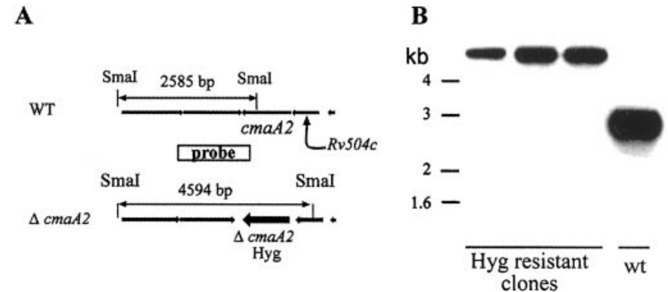

FIG. 2. **Construction of a deletion mutation of *cmaA2* in *M. tuberculosis* by specialized transduction.** *A,* map of the *cmaA2* genomic region in both wild-type and *cmaA2* mutant strains. *B,* Southern blot of *Sma*I-digested genomic DNA from the indicated strain probed with the fragment indicated in *A*. All three hygromycin-resistant strains contain the *cmaA2* disruption.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Wild-type *M. tuberculosis* Erdman is a stock of an animal-passaged strain that has been passaged once *in vitro*. *M. tuberculosis* strain Erdman was grown at 37 °C in 7H9 (broth) or 7H10 (agar) (Difco) media with OADC enrichment (Becton Dickinson), 0.5% glycerol, 0.05% Tween 80 (broth), and where appropriate, hygromycin (Roche Molecular Biochemicals) at 50 μg/ml or kanamycin (Sigma) 20 μg/ml. The *M. tuberculosis* Erdman strain with the Δ*cmaA2*::*hyg* allele is designated mc^{2}3120. For mycolic acid analysis, the wild-type strain was wild-type Erdman transformed with pmv306 hygro, an integrating vector that supplies a single copy hygromycin resistance gene.

Disruption of cmaA2 and Complementation—A Δ*cmaA2*::*hyg* allele was constructed by amplifying the flanking regions of the *cmaA2* gene and inserting these fragments on either side of the hygromycin resistance gene. Specifically, a 619-bp flanking region of *cmaA2* 5′ to the start codon was amplified by PCR using primers omsg33 and omsg34, which contain *Xba*I and *Asp*7181 sites at their respective 5′-termini. A 646-bp flanking region 3′ to the stop codon was amplified using primers omsg35 and omsg36, which introduce *Hin*dIII and *Spe*I sites, respectively. The PCR products were cloned, sequenced, and inserted flanking the hygromycin cassette in pMSG284, a cloning vector containing a bacteriophage lambda *cos* site, a *Pac*I site, and the hygromycin resistance gene flanked by resolvase sites. The final knockout construct (pMSG104) was packaged into phAE87 as previously described (6) to create phMSG104. PhMSG104 was used to transduce wild-type *M. tuberculosis* to hygromycin resistance as previously described (6).

For complementation, an *M. tuberculosis* Erdman cosmid library was screened for *cmaA2*-containing clones by PCR. Cosmid 3E4 was digested with *Xba*I/*Nco*I and a 2093-bp fragment containing *Rv504c*, *cmaA2*, and part of *Rv502* was cloned into pmv206 hyg to create pMSG129. To create an inframe deletion of *Rv504c*, the 1522-bp *Bst*EII/*Hin*dIII fragment, the 2279-bp *Bst*EII/*Mlu*I fragment, and the 2203-bp *Mlu*I/*Hin*dIII fragment from pMSG129 were isolated after creating blunt ended *Bst*EII ends with the Klenow fragment and were ligated in a three piece ligation to create pMSG133. Using this strategy, *Rv504c* was reduced to a truncated fusion protein of 81 amino acids. The reading frame of the fusion joint was confirmed by DNA sequencing. To create single copy complementation constructs, the inserts of pMSG129 and pMSG133 were subcloned into pmv306kan, a site-specific integrating mycobacterial vector (12) to create pMSG134 and pMSG136, respectively.

Expression of cmaA2 in M. smegmatis—*M. smegmatis* strain mc^2 155 was transformed with pMSG129 or vector control and total mycolic acids that were prepared as described below. Total mycolic acids were examined by proton NMR for the presence of *cis*- or *trans*-cyclopropane residues. For coexpression of *cmaA2* with *mmaA1*, the *mmaA1* open-reading frame with its putative promoter (13) was cloned as a 1056-bp *NgoM*IV/*Avr*II fragment into pMSG137 digested with *NgoM*IV/*Nhe*I to create pMSG148.

Preparation and Analysis of Mycolic Acids—For radiolabeled mycolic acids, 50 ml of mid-log phase liquid cultures were incubated with 50 μCi of [^{14}C]acetate (PerkinElmer Life Sciences) for 12–18 h. Total mycolic acid methyl esters were prepared as described previously (6) and precipitated with toluene/acetonitrile. Analytical and preparative TLC was performed as previously described (6), and radio TLCs were analyzed on a phosphorimager cassette (Molecular Dynamics).

NMR Spectroscopy—One-dimensional ^{1}H NMR spectra were acquired at 27 °C on either a Bruker DRX300 or DRX600 spectrometer in deuterochloroform (Cambridge Isotope Labs) and were referenced to the chloroform peak. Two-dimensional DQF-COSY and TOCSY NMR experiments were performed at 27 °C on a Bruker DRX600 spectrometer equipped with a 5 mm TXI probe. Typically, 256 T_1 increments, each with 64 scans and 4000 data points over a spectral width of 5 kHz, were collected for each spectrum. The two-dimensional TOCSY experiment employed a 100 ms MLEV17 mixing sequence with a 9kHz spinlock field. Data processing and analysis was performed using Bruker XWIN-NMR software.

Sequence Analysis—Sequence alignment and phylogenetic tree construction was performed as described (14) on the Multalin server.

RESULTS

Inactivation of cmaA2 in M. tuberculosis by Allelic Exchange and Complementation with Wild-type cmaA2—To define the function of *cmaA2* in *M. tuberculosis,* we sought to delete *cmaA2* from the chromosome of the Erdman strain of *M. tuberculosis* by allelic exchange. We constructed a substrate for allelic exchange at *cmaA2* by replacing the coding region with a hygromycin resistance gene as described under "Experimental Procedures." We packaged this knockout construct into a specialized transducing mycobacteriophage and infected wild-type *M. tuberculosis* as previously described. (6, 16).[2] Antibiotic-resistant *M. tuberculosis* clones were screened for allelic exchange at *cmaA2* by Southern blotting. Three hygromycin-resistant clones contained the *cmaA2* disruption (Fig. 2*B*), and one was designated mc^{2}3120 and used for further studies.

To show that any phenotype observed for the *cmaA2* mutant was attributable to the *cmaA2* mutation, we complemented the *cmaA2* mutant with *cmaA2* in single copy under its own promoter. Inspection of the genomic sequence surrounding *cmaA2* suggests that this gene is transcribed as the second gene in a two gene operon with *Rv504c*, a gene of unknown function (see Fig. 2*A* for diagram). To complement the *cmaA2* mutant with only *cmaA2* under its native promoter, we reconstructed the *cmaA2* operon with an inframe deletion in *Rv504c* and complemented the *cmaA2* mutant in single copy with this inframe deletion construct (pMSG136). The strains mc^{2}3120 and mc^{2}3120 (pmsg136) were analyzed in the subsequent experiments.

Inactivation of cmaA2 Alters the Oxygenated Mycolic Acids of M. tuberculosis—As shown previously for *pcaA* (6) in the absence of a cyclopropane synthetase, the mycolic acids of *M. tuberculosis* would likely acquire an unsaturation. Therefore, we examined [^{14}C]acetate-labeled mycolic acids of the *cmaA2*

[2] S. Bardarov, M. Larsen, M. Pavelka, S. S. Bardarov, Jr., and W. R. Jacobs, manuscript in preparation.

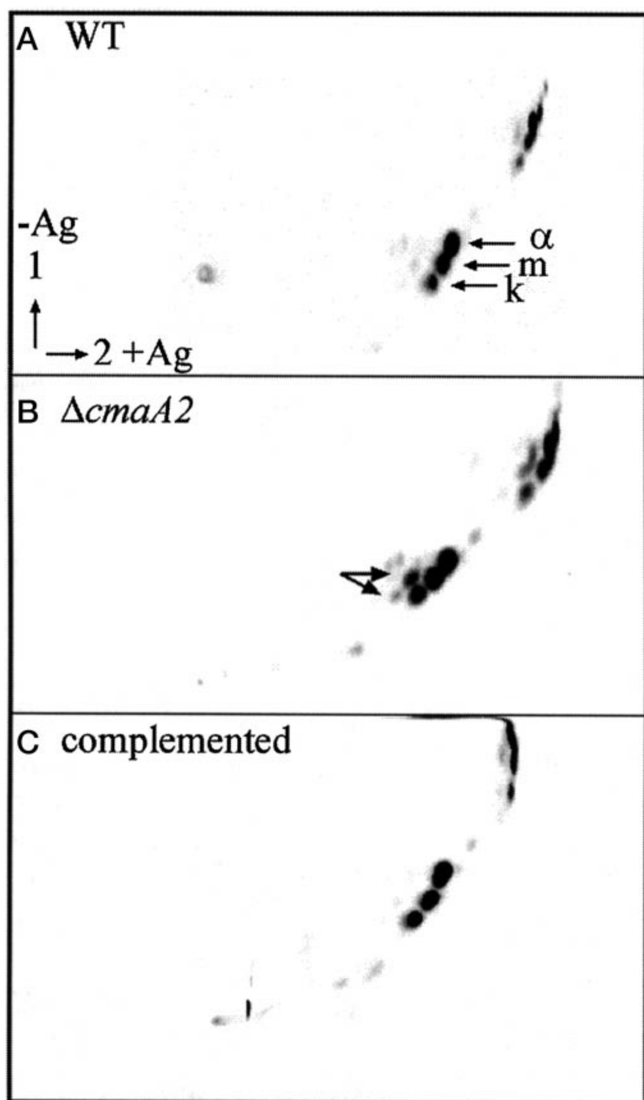

FIG. 3. **Radio two-dimensional TLC analysis of *cmaA2* mutant mycolic acids.** *A,* TLC system is described in detail in the text. The sample is developed along the left edge without silver impregnation (*arrow 1* in *A*) and then in the second dimension with silver impregnation (*arrow 2* in *A*). ^{14}C-labeled mycolates from wild-type *M. tuberculosis* Erdman (*A*), *M. tuberculosis* Δ*cmaA2*::*hyg* (*B*), and *M. tuberculosis* Δ*cmaA2*::*hyg attB*::pMSG136 (*cmaA2*) (*C*) are shown. The *arrows* in *B* point to new mycolic acid species with the polarity of methoxy- and ketomycolates that are retarded in the silver dimension

mutant by two-dimensional argentation TLC. This TLC system has been described previously for the analysis of *M. tuberculosis* mycolic acids (6, 11). Briefly, the TLC plate is impregnated with silver nitrate leaving an unimpregnated strip along the left edge. The sample is developed first along the unimpregnated strip to separate the mycolates by polarity (Fig. 3*A, arrow 1*). The plate is then developed in the silver dimension (Fig. 3*A, arrow 2*), separating the mycolates by degree of unsaturation. Silver nitrate retards the migration of unsaturated lipids relative to saturated or cyclopropanated lipids. Therefore, in the absence of a cyclopropane synthetase, an unsaturated mycolic acid retarded in the second dimension might appear.

The TLC pattern of wild-type *M. tuberculosis* mycolates is shown in Fig. 3*A*. The α-, methoxy-, and ketomycolates are labeled and correspond to the structures given in Fig. 1. *cis*- and *trans*-Cyclopropanated oxygenated mycolates are not distinguished in this TLC system. Inactivation of *cmaA2* alters the oxygenated mycolic acids. Specifically, two new mycolic acid species are visible in the *cmaA2* mutant with the polarity of methoxy- and ketomycolates but which are retarded by silver impregnation (Fig. 3*B*). The α-mycolate of the *cmaA2* mutant is identical to that from wild-type in its mobility. To demonstrate that this phenotype is due specifically to the loss of *cmaA2*, we examined the mycolic acids from the complemented mutant. Wild-type mycolic acid patterns were restored in the complemented strain, demonstrating that the altered oxygenated mycolates are secondary to the *cmaA2* mutation (Fig. 3*C*). Thus, inactivation of *cmaA2* causes the accumulation of an unsaturated subpopulation of oxygenated mycolates, demonstrating that *cmaA2* is required for the proper cyclopropanation of these lipids.

Inactivation of cmaA2 Abolishes trans-Cyclopropanated Mycolates—Because the *cmaA2* mutant has defects in a subpopulation of oxygenated mycolates, we reasoned that *cmaA2* may be involved in either the *cis* or *trans* cyclopropanation of these molecules. To define the mycolic acid alteration in the *cmaA2* mutant, we examined total mycolic acids from wild-type and the *cmaA2* mutant by ^{1}H NMR, a technique that can clearly distinguish between *cis*- and *trans*-cyclopropane residues. The *cis*- and *trans*-cyclopropane proton resonances contributed by the three mycolic acid classes of wild-type *M. tuberculosis* are visible in the region of the NMR spectrum shown in Fig. 4*A*, *top panel* (3). In this expansion of the region from −0.4 ppm to 0.8 ppm, the characteristic resonances of *cis*-cyclopropane hydrogens (−0.33 ppm 2H, 0.56 ppm 1H) and *trans*-cyclopropane hydrogens (0.15 ppm 2H, 0.45 ppm 1H) can be distinguished (Fig. 4*A*, *cis*- and *trans*-cyclopropane structures label corresponding peaks). The *cis*-cyclopropane proton peak at 0.67 ppm (1H) and the *trans*-cyclopropane proton peak at 0.70 ppm are overlapping. In the wild-type Erdman strain used in this study, the ratio of *cis*/*trans* cyclopropane hydrogens is 8:1, lower than in previously examined laboratory strains (13).

The *cmaA2* mutant lacks *trans*-cyclopropane rings, as evidenced by the complete absence of the complex mutiplets at 0.15 and 0.45 ppm in the spectrum shown in Fig. 4*B*. Importantly, the *cis*-cyclopropane resonances are unaffected. The TLC data presented above demonstrates that the oxygenated mycolates in the *cmaA2* mutant contain a subpopulation of unsaturated mycolates. Accordingly, the NMR spectrum of the total mycolates from the *cmaA2* mutant contains a complex multiplet at 5.33 ppm that is not present in wild-type mycolates (Fig. 4, *A* and *B*), consistent with the presence of the unsaturated mycolates in the mutant strain.

To further investigate the structure of the altered oxygenated mycolic acids in the *cmaA2* mutant, we examined the mycolic acids of wild-type and mutant strains by two-dimensional COSY and TOCSY proton NMR spectroscopy. We first confirmed the previously reported structure of the cyclopropyl groups and their surrounding functional groups in total mycolic acids from wild type (Fig. 4*D*). According to the two-dimensional TOCSY spectrum, the *cis*-cyclopropyl hydrogen resonances at −0.33, 0.56, and 0.67 ppm all belong to the coupled spin network, as do the *trans*-cyclopropyl hydrogen resonances at 0.15, 0.45, and 0.7 (Fig. 4*D*). In addition, the *trans*-cyclopropyl group protons are adjacent to a methyl branch, as evidenced by a TOCSY cross-peak between the *trans*-cyclopropane proton resonances and a doublet at 0.95 ppm (Fig. 4*D*).

Two-dimensional TOCSY spectroscopy of purified methoxymycolates from the *cmaA2* mutant confirmed the lack of *trans*-cyclopropyl protons demonstrated on the one-dimensional spectrum (Fig. 4*E*). In addition, the unsaturated derivatives of the methoxymycolates seen on TLC contain predominantly *trans* double bonds, as evidenced by the TOCSY cross-peak between

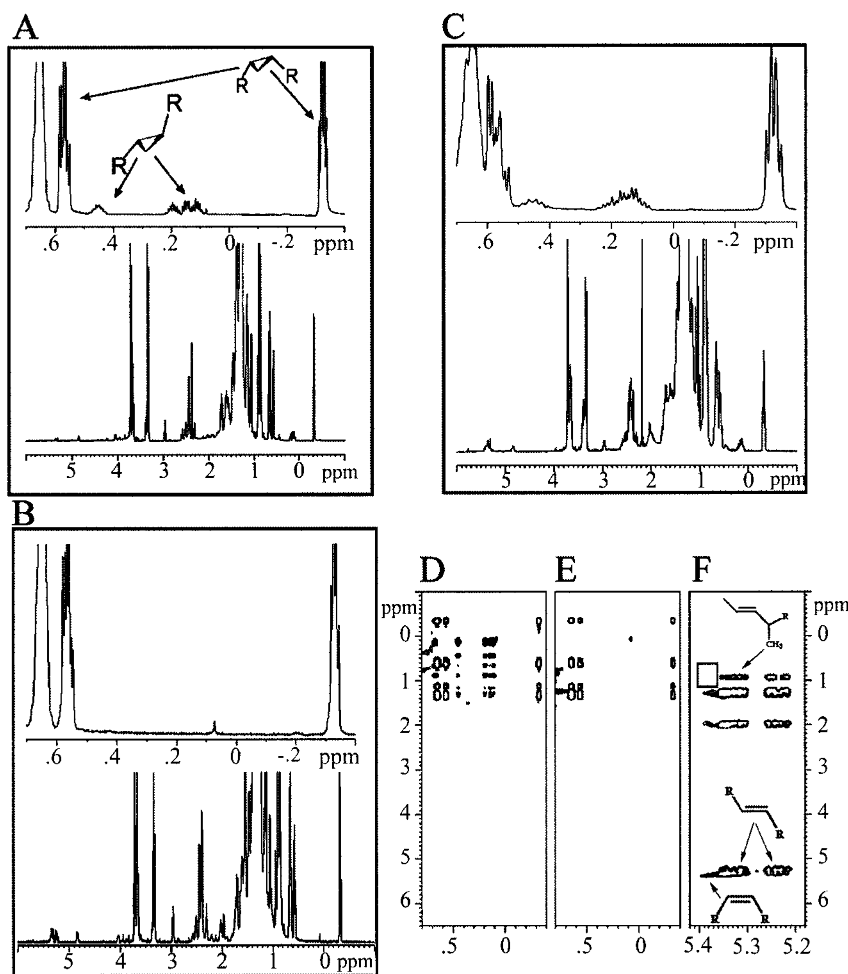

FIG. 4. **^{1}H NMR analysis of wild-type and *cmaA2* mutant mycolic acids.** One-dimensional ^{1}H NMR from wild type (*A*), *cmaA2* mutant (*B*), and complemented mutant (*C*). The *lower panels* show the entire spectrum whereas the *upper panels* are expansions of the region from −0.4 to 1 ppm, demonstrating the *cis*- and *trans*-cyclopropyl protons that are labeled with structures in *A*. *D,* two-dimensional TOCSY spectrum showing the region from −0.4 to 1 ppm demonstrating the *cis*- and *trans*-cyclopropyl structures from wild-type total mycolic acids. *E* and *F,* two-dimensional TOCSY spectra on purified methoxymycolates from the *cmaA2* mutant. *E* shows the same region as *D* and demonstrates the lack of *trans*-cyclopropyl protons in the mutant. *F* shows the TOCSY connections between the *trans*-vinyl protons centered at 5.33 ppm and the allylic methyl branch at 0.95 ppm, the neighboring methine proton (2.0 ppm), and the adjacent CH_2 groups (1.3 ppm). The vinyl proton resonance at 5.39 ppm does not show a connection with the methyl branch (*boxed area* in *F*), demonstrating that the unsaturated methoxymycolates in the *cmaA2* mutant contain a subpopulation with a *cis* double bond.

the vinyl proton resonance centered at 5.33 ppm and the methyl branch resonance at 0.95 ppm (Fig. 4*F* and Ref. 8) and a COSY cross-peak between the vinyl protons and a methine proton resonance at 2 ppm (data not shown). *cis*-Cyclopropanes and *cis* double bonds in mycolic acids are not adjacent to methyl branches. Accordingly, a weak resonance at 5.39 ppm does not show a TOCSY cross-peak with the methyl branch at 0.95 ppm, indicating a small amount of *cis*-unsaturated methoxymycolate (Fig. 4*F*, *box* at 0.95 ppm) in the *cmaA2* mutant methoxymycolates.

The *trans* cyclopropanation defect in the *cmaA2* mutant was somewhat surprising as *cmaA2* had previously been shown to catalyze the formation of *cis*-cyclopropane rings when overexpressed in *M. smegmatis* (11). Therefore, we considered whether the lack of *trans*-cyclopropane residues in the *cmaA2* mutant could be an indirect effect on another, as yet undefined, cyclopropane synthetase. To investigate this possibility, we purified individual mycolate classes of the *cmaA2* mutant by preparative TLC and examined them by proton NMR. Individual mycolate classes were examined for the presence of cyclopropane and methyl branch resonances known or likely to be added by the SAM-dependent methyl transferases of *M. tuberculosis*. The α-mycolate of the *cmaA2* mutant was identical to wild-type α-mycolate (data not shown). As detailed above, the methoxymycolate of the *cmaA2* mutant exhibited characteristic resonances of *cis*-cyclopropane protons, methyl branch protons adjacent to a methoxyl group (0.85 ppm, doublet), and the allylic methyl branch of the proximal *trans* double bond (0.95 ppm, doublet, Ref. 8). The ketomycolate also contained all predicted resonances except for the *trans*-cyclopropane residues. Therefore, as assessed by proton NMR of individual mycolate classes from the *cmaA2* mutant, the only cyclopropane or methyl branch missing from the mycolic acids of the mutant is the *trans*-cyclopropane ring.

Expression of cmaA2 in M. smegmatis—The data presented above show that *cmaA2* is the *trans*-cyclopropane synthetase of *M. tuberculosis.* To confirm that *cmaA2* produces *cis*-cyclopropane rings in *M. smegmatis* as had been previously reported (11), we introduced *cmaA2* into *M. smegmatis* on a multicopy plasmid under its own promoter. NMR examination of total mycolic acids from this strain revealed *cis*-cyclopropane proton resonances but not *trans*-cyclopropane proton resonances (data not shown). Mmas1 appears to catalyze the isomerization of the proximal *cis* double bond in oxygenated mycolates with the introduction of an allylic methyl branch (13). As this isomerization is necessary for *trans*-cyclopropane formation, we investigated whether *cmaA2* would produce *trans*-cyclopropane rings in *M. smegmatis* when introduced with *mmaA1*. When coexpressed with *mmaA1*, *cmaA2* still catalyzed only *cis*-cyclopropane formation (data not shown).

DISCUSSION

The mycolic acid methyl transferases of *M. tuberculosis* are a large family of highly homologous proteins that modify the mycolic acids of the cell wall with cyclopropane rings and methyl branches. Although cyclopropanated fatty acids are found in many bacteria (17), *M. tuberculosis* has evolved an elaborate enzymatic system of cyclopropane synthetases not found in any other bacteria. In this work we have shown that one of these transferases, *cmaA2,* is a *trans*-cyclopropane synthetase for oxygenated mycolates and that the other members of this gene family cannot compensate for the loss of *cmaA2*.

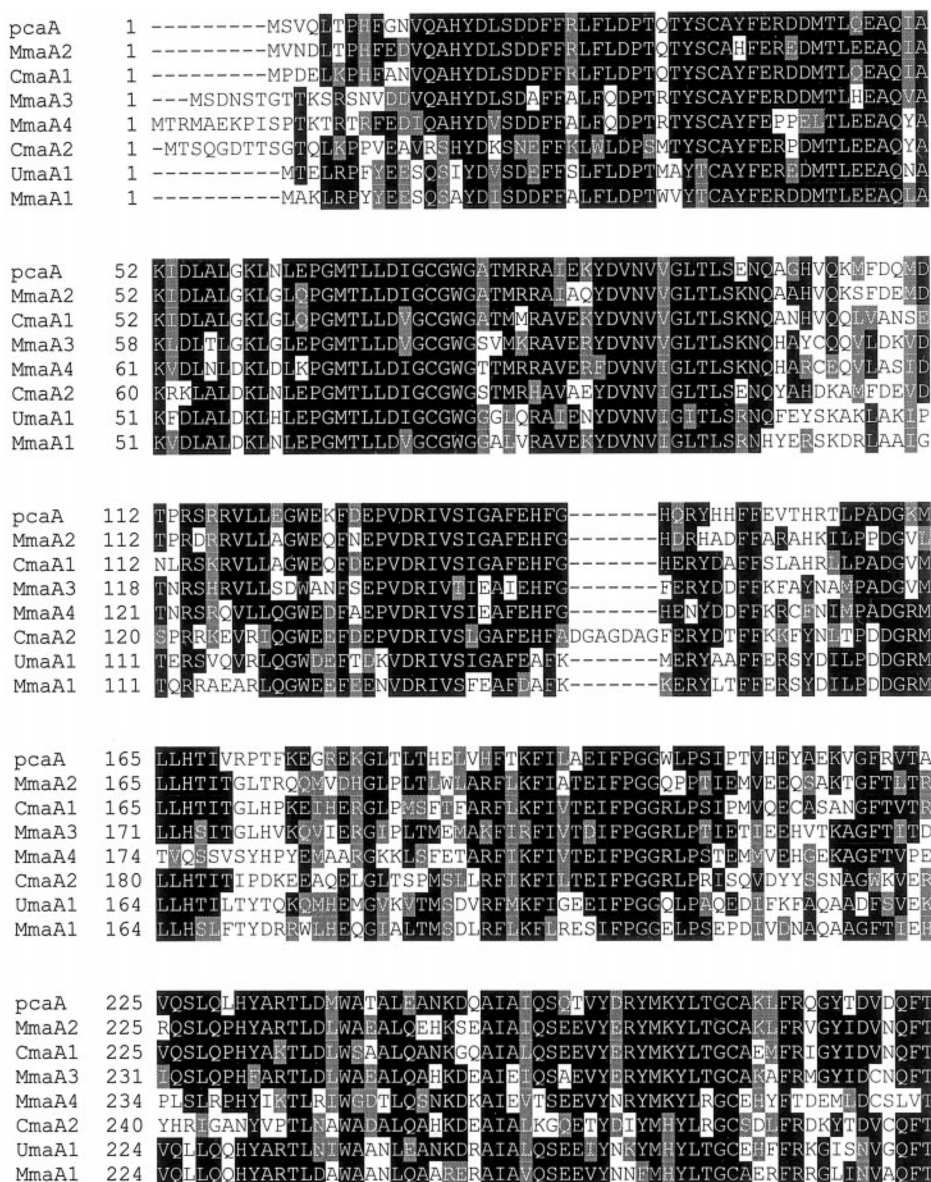

FIG. 5. **Amino acid sequence alignment and phylogenetic tree of the putative mycolic acid methyl transferases of *M. tuberculosis.*** *Black* or *shaded residues,* identical amino acids.

All of the members of this gene family share striking amino acid sequence similarity. The sequence alignment of these proteins shown in Fig. 5 demonstrates that the individual cyclopropane synthetases share substantial amino acid identity over most of their length and that the sequence divergence between the members is limited to several distinct regions. Despite this striking sequence conservation, each member of this gene family appears to have a distinct catalytic function that cannot be compensated by another member of the family. Specifically, we have shown previously that inactivation of *pcaA* abolishes proximal cyclopropanation of the α-mycolate molecule despite intact *cmaA2*, *mmaA2*, and *cmaA1* genes. It is interesting to note in the sequence alignment that CmaA2 contains an 8-amino acid segment at amino acids 152–160 that is not present in any of the other methyl transferases. As CmaA2 is the only *trans*-cyclopropane synthetase of the group, this eight amino acid segment may be important for catalysis or substrate binding. In addition, a phylogenetic tree derived from these sequences demonstrates that there are three distinct groups within this gene family that are consistent with the known or

suspected functions of these proteins (Fig. 5). The first group contains MmaA3 and MmaA4, two proteins that introduce the methoxy group in the distal position of the methoxymycolates (7–9, 18, 19). The second group contains CmaA2, MmaA1, and UmaA1. MmaA1 is likely responsible for the isomerization of the proximal *cis* double bond to a *trans* double bond in the meromycolate chain with simultaneous introduction of an allylic methyl branch (13). Because overexpression of MmaA1 in *M. tuberculosis* produced an excess of both *trans* unsaturated and *trans*-cyclopropanated mycolic acids, MmaA1 action is presumably an early step in *trans*-cyclopropane synthesis. It is therefore logical that CmaA2 is within the same phylogenetic subfamily. UmaA1 has no known function at present. The last group contains PcaA, CmaA1, and MmaA2. All of these enzymes are known or putative *cis*-cyclopropane synthetases. PcaA synthesizes the proximal *cis*-cyclopropane ring of the α-mycolates (6), CmaA1 produces a distal *cis*-cyclopropane ring in the α-mycolate of *M. smegmatis* (15), and MmaA2 likely synthesizes the proximal *cis*-cyclopropane ring of the methoxymycolates (8, 9). Three-dimensional structural studies of these proteins may help elucidate the basis for their substrate specificity.

Several explanations are possible for the ability of *cmaA2* to produce *cis*-cyclopropanes in *M. smegmatis*. Given the high sequence identity within this gene family, it is possible that *cmaA2* can inefficiently catalyze *cis*-cyclopropane synthesis when highly overexpressed. Alternatively, the substrate specificity of these enzymes may be determined in part by physical association in multienzyme complexes. Although this possibility has not been examined experimentally, these enzymes catalyze the sequential modification of the meromycolate chain of mycolic acids and therefore could associate in multienzyme complexes to achieve efficient modification of a mycolic acid subclass. Therefore, it is possible that in *M. smegmatis,* CmaA2 cannot associate with other methyl transferases and the correct CmaA2 substrate is not available.

The significance of *trans*-cyclopropanated oxygenated mycolic acids for *M. tuberculosis* pathogenesis is unknown. However, previous work has shown that clinical strains of *M. tuberculosis* have higher *trans*-cyclopropane content than extensively passaged laboratory strains, suggesting that *in vivo* growth either dynamically enhances *trans*-cyclopropane formation or favors subpopulations of *M. tuberculosis* with higher *trans*-cyclopropane content (13). These results are consistent with the high proportion of *trans*-cyclopropane rings in the wild-type *M. tuberculosis* strain used in this study as this strain was recently passaged through animals and has not been passaged significantly *in vitro*. The results presented here define *cmaA2* as the *trans*-cyclopropane synthetase of *M. tuberculosis.* Further examination of the *cmaA2* mutant in animal models of infection will broaden our understanding of the role of individual cyclopropane residues in general, and of *trans*-cyclopropane residues in particular, in *M. tuberculosis* pathogenesis.

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