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

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 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-, β-, and γ-mycolates (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.
cmaA2 Encodes a trans-Cyclopropane Synthetase

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), 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 ΔcmaA2::hyg allele is designated mc²3120. For mycolic acid analysis, the wild-type strain was wild-type Erdman transformed with pMSG137 digested with an appropriate, hygromycin (Roche Molecular Biochemicals) at 50 μg/ml or kanamycin (Sigma) 20 μg/ml. The M. tuberculosis Erdman strain with the ΔcmaA2::hyg allele is designated mc²3120. For mycolic acid analysis, the wild-type strain was wild-type Erdman transformed with pMSG137 digested with

Disruption of cmaA2 and Complementation—A ΔcmaA2::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 XbaI and Asp7181 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 HindIII and SphiI 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 Puc1 site, and the hygromycin resistance gene flanked by resolvase sites. The final knock out construct (pMSG104) was packaged into phAE87 as previously described (6) to create pMSG104. 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 XbaI/AscI and a 1206-bp fragment containing Rv504c, cmaA2, and part of Rv502 was cloned into pMV306 hygro, an integrating vector that supplies a single copy hygromycin resistance gene under its native promoter. The resulting plasmid, pMSG133, was transformed with pMSG129 or vector control and total mycolic acids were prepared as described below. Total mycolic acids were analyzed by proton NMR for the presence of cis- or trans-cyclopropane residues. For coexpression of cmaA2 with nmaA1, the nmaA1 open-reading frame with its putative promoter (15) was cloned as a 1056-bp NgoMIV/NheI fragment into pMSG157 digested with NgoMIV/NheI 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 [14C]acetate (PerkinElmer Life Sciences) for 12–18 h. Total mycolic acid methyl esters were prepared as described previously (6) and precipitated with toluene/acetone. 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 1H NMR spectra were acquired at 27 °C on either a Bruker DRX300 or DRX600 spectrometer in deuterchloroform (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 T₁ 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 9 kHz 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 chromosomes 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). Antibiotic-resistant M. tuberculosis clones were screened for allelic exchange at cmaA2 by Southern blotting. Three hygromycin-resistant clones contained the cmaA2 disruption (Fig. 2B), and one was designated mc²3120 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. 2A for diagram). To complement the cmaA2 mutant with only cmaA2 under its native promoter, we reconstructed the cmaA2 operon with this deletion construct (pMSG136). The strains mc²3120 and mc³3120 (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 [14C]acetate-labeled mycolic acids of the cmaA2

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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. 3B). The a-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. 3C). 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 Mycobacterium tuberculosis are visible in the region of the NMR spectrum shown in Fig. 4A, 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. 4A, 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 multiplets at 0.15 and 0.45 ppm in the spectrum shown in Fig. 4B. 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. 4D). 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. 4D). 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. 4D).

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. 4E). In addition, the unsaturated derivatives of the methoxymycolates seen on TLC contain predominantly trans double bonds, as evidenced by the TOCSY cross-peak between
the vinyl proton resonance centered at 5.33 ppm and the methyl branch resonance at 0.95 ppm (Fig. 4F 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, demonstrating that the unsaturated methoxymycolates in the cmaA2 mutant contain a subpopulation with a cis double bond.

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 from 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 kmcmA 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). MmA1 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.
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 \textit{pcaA} abolishes proximal cyclopropanation of the α-mycolate molecule despite intact \textit{cmaA2}, \textit{mmaA2}, and \textit{cmaA1} genes. It is interesting to note in the sequence alignment that \textit{cmaA2} contains an 8-amino acid segment at amino acids 152–160 that is not present in any of the other methyl transferases. As \textit{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
contains MmaA3 and MmaA4, two proteins that introduce the suspected functions of these proteins (Fig. 5). The first group contains MmaA3 and MmaA4, two proteins that introduce the.

MmaA1 is likely responsible for the isomerization of mycolates (8, 9). Three-dimensional structural studies of these enzymes are known or putative cis-cyclopropane synthetases. 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. CmaA2 Encodes a trans-Cyclopropane Synthetase. 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 mycoloyl branch (13). Because overexpression of MmaA1 in M. tuberculosis produces 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 mycoloyl branch (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.

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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