

A Novel Mycolic Acid Cyclopropane Synthetase Is Required for Cording, Persistence, and Virulence of *Mycobacterium tuberculosis*

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Summary

Colonial morphology of pathogenic bacteria is often associated with virulence. For *M. tuberculosis*, the causative agent of tuberculosis (TB), virulence is correlated with the formation of serpentine cords, a morphology that was first noted by Koch. We identified a mycobacterial gene, *pcaA*, that we show is required for cording and mycolic acid cyclopropane ring synthesis in the cell wall of both BCG and *M. tuberculosis*. Furthermore, we show that mutants of *pcaA* fail to persist within and kill infected mice despite normal initial replication. These results indicate that a novel member of a family of cyclopropane synthetases is necessary for lethal chronic persistent *M. tuberculosis* infection and define a role for cyclopropanated lipids in bacterial pathogenesis.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), killed approximately two million people worldwide in 1997 and has infected approximately 32% of the world's population (Dye et al., 1999). Despite decades of intense and productive investigation, the molecular mechanisms of *M. tuberculosis* pathogenesis are still largely unknown (Wilson et al., 1995; Berthet et al., 1998; Li et al., 1998). Much speculation concerning virulence determinants of *M. tuberculosis* has focused on the unique chemical entities found in the cell envelope of this gram-positive organism (Brennan and Nikaido, 1995; Barry et al., 1998; Daffe and Draper, 1998). The cell envelope of *M. tuberculosis* is a highly complex hydrophobic array of unique glycolipids and mycolic acids. Mycolic acids are very long-chain branched fatty acids that are found either covalently attached to the cell wall or in the form of trehalose dimycolate (TDM), a toxic glycolipid of *M. tuberculosis* (see Figure 4A for schematic diagram). Although an impressive body of structural and chemical information has accumulated about the constituents of the cell wall of *M. tuberculosis*, definitive understanding of the pathogenetic role of the cell envelope in general, and of mycolic acids in particular, has been limited by a lack of mutants of *M. tuberculosis* defective in the synthesis of specific cell surface

molecules. Recently, advances in the genetics of *M. tuberculosis* have allowed more facile manipulation of this once genetically intractable organism (Jacobs et al., 1987; Bardarov et al., 1997; Pelicic et al., 1997). These advances, in combination with the recent completion of the *M. tuberculosis* genome sequence, have facilitated further research into the contribution of individual genes to *M. tuberculosis* virulence (Cole et al., 1998; Cox et al., 1999).

Virulent strains of pathogenic bacteria frequently exhibit colony morphologies that correlate with their virulence and that reflect the importance of cell wall chemical structure in the pathogenesis of many bacterial diseases. These morphologies often reflect subtle biochemical changes at the cell surface that can dramatically alter virulence (Austrian, 1953; Stern et al., 1986; Guo et al., 1998). In his original description of *M. tuberculosis*, Koch noted that the bacteria formed braided microscopic bundles, a morphology that was later termed cording (Koch, 1882). Further studies demonstrated that the virulence of an individual mycobacterial strain for mice could be predicted by quantitating the strength of its cording, observed either microscopically or by colonial morphology (Middlebrook et al., 1947; Darzins and Fahr, 1956; Pierce et al., 1956). Because cording is likely the result of cell surface interactions, the association between cording and virulence supports the idea that the cell envelope of *M. tuberculosis* is important for pathogenesis. The cell surface components responsible for cording have not been defined.

Cyclopropane rings are a modification of membrane lipids that are found in diverse bacterial species but only a limited number of eukaryotes. The cyclopropane fatty acid synthase (CFAS) of *Escherichia coli* has been extensively studied and is an S-adenosyl methionine-dependent methyl transferase that introduces cyclopropane rings into unsaturated fatty acids in the cell membrane (Grogan and Cronan, 1997). *M. tuberculosis* synthesizes three major types of cyclopropanated mycolic acids through the action of five putative homologous cyclopropane synthetases, some of which have been shown to have mycolic acid cyclopropanating activity when introduced into *M. smegmatis*, a saprophytic mycobacterial species that does not produce cyclopropanated mycolic acids (George et al., 1995; Yuan et al., 1995; Yuan and Barry, 1996; Dubnau et al., 1997). Despite the presence of such an elaborate enzymatic system for the synthesis of cyclopropanated mycolic acids, knowledge of the physiological function of this membrane modification is limited. Although cyclopropanated mycolic acids are presumed to be important in the pathogenesis of TB based on their abundance and the amount of metabolic energy devoted to their synthesis, their specific role remains to be defined.

Given the relationship between cording and virulence, we reasoned that elucidating the genetic basis for cording would yield insights into the role of the cell envelope in mycobacterial virulence. BCG is an attenuated strain of *M. bovis* that is widely used as a vaccine for TB. Although attenuated as a result of multiple genomic

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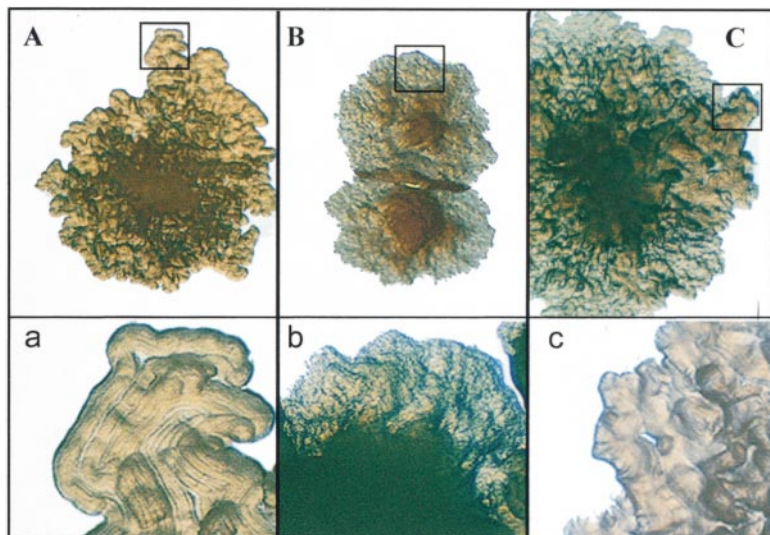


Figure 1. *pcaA* Is Involved in Colonial Cording in BCG Pasteur

Lower panels (lowercase letters) are higher magnification views of the boxed areas in the single colonies shown in upper panels (capital letters). All colonies grown on cord reading agar as described in Experimental Procedures and photographed at the same magnification.

(A and a) BCG Pasteur.

(B and b) BCG Pasteur *pcaA*::*Tn5370*.

(C and c) BCG Pasteur *pcaA*::*Tn5370 attB*::*pcaA*.

deletions, BCG retains the ability to replicate within humans and mice and exhibits cording (Middlebrook et al., 1947; Behr et al., 1999). Therefore, we undertook a genetic screen for mutants of BCG that failed to form wild-type cords. Through this approach, we defined the function of a novel mycobacterial gene, *pcaA*, which we show is required for synthesis of the proximal cyclopropane ring of the major mycolic acid of both BCG and *M. tuberculosis*. The *M. tuberculosis* mutant of *pcaA* replicates normally in vivo but fails to persist within and kill infected mice. These results define cyclopropane synthetases as a novel class of persistence genes necessary for lethal chronic *M. tuberculosis* infection.

Results

Screening a BCG Transposon Library for Mutants Deficient in Cord Formation

When grown on solid media (Lorian, 1966, 1969), BCG exhibits a colony morphology consisting of serpentine ridges throughout the colony that are oriented parallel to the axis of larger bundles of organisms forming macroscopic linear “cords” or ropes (Figure 1A). The individual cell contacts characteristic of cording can be assayed by microscopic examination of acid fast stained cells. As illustrated in Figure 2A, BCG Pasteur forms microscopic cords that consist of bacteria aligned along their long axes in a parallel array.

To investigate the genetic basis for the cording phenotype in slow-growing mycobacteria, we generated a transposon library of BCG Pasteur, visually screened for mutants with altered colony morphologies, and confirmed their cording morphologies by microscopic examination. Not all colony morphologic variants were selected for further analysis, only those that were specifically lacking the serpentine ridges demonstrated in Figure 1. Out of approximately 3500 colonies screened, three mutants were deficient for cord formation as assessed by colony morphology and microscopic examination. One of these mutants, mc²2801, displayed an altered colonial and microscopic cording phenotype as compared to wild-type BCG. In mc²2801, the serpentine ridges present in wild-type colonies are replaced by

a grainy surface without visible bundles of organisms (compare Figures 1A and 1a and Figures 1B and 1b). In addition, mc²2801 displays altered microscopic cording, forming disordered aggregates without the tightly packed cords visible in wild type (compare Figures 2A and 2B).

The transposon in mc²2801 interrupts a gene of unknown function that is highly homologous to a *M. tuberculosis* gene that has been designated *umaA2* in the *M. tuberculosis* genome annotation. *UmaA2* is part of a family of methyltransferases that synthesize cyclopropane rings on the mycolic acids of the cell wall of *M. tuberculosis* (Barry et al., 1998). The protein sequence of *UmaA2* of *M. tuberculosis* shares 68% amino acid identity and 77% similarity with the *M. tuberculosis* cyclopropane synthase *cmaA1* (Yuan et al., 1995) and 33% identity/46% similarity with the CFAS of *E. coli*. We have renamed this gene *pcaA* based on its function defined below.

Inactivation of *pcaA* Affects Cording in *M. tuberculosis*

To confirm that the altered cording phenotype associated with inactivation of *pcaA* in BCG was applicable to *M. tuberculosis*, we deleted the *pcaA* gene from the genome by homologous recombination. Allelic exchange in *M. tuberculosis* has been technically prohibitive in the past and only achievable through laborious and time-consuming counterselection procedures (Balasubramanian et al., 1996; Pelicic et al., 1997; Pavelka and Jacobs, 1999). To disrupt *pcaA*, we utilized a recently developed phage-mediated system to deliver the knockout construct into *M. tuberculosis* cells (Figure 3A). This approach has been used successfully to achieve efficient transposon mutagenesis of *M. tuberculosis* (Bardarov et al., 1997) and has recently been successfully employed in our laboratory to achieve efficient allelic exchange in BCG. *M. tuberculosis* Erdman was infected with a recombinant phage designed to delete the entire open reading frame of *pcaA*. Hygromycin-resistant colonies were screened for correct allelic exchange at *pcaA* by PCR and confirmed by Southern blotting (Figure 3B). By both screening techniques, four

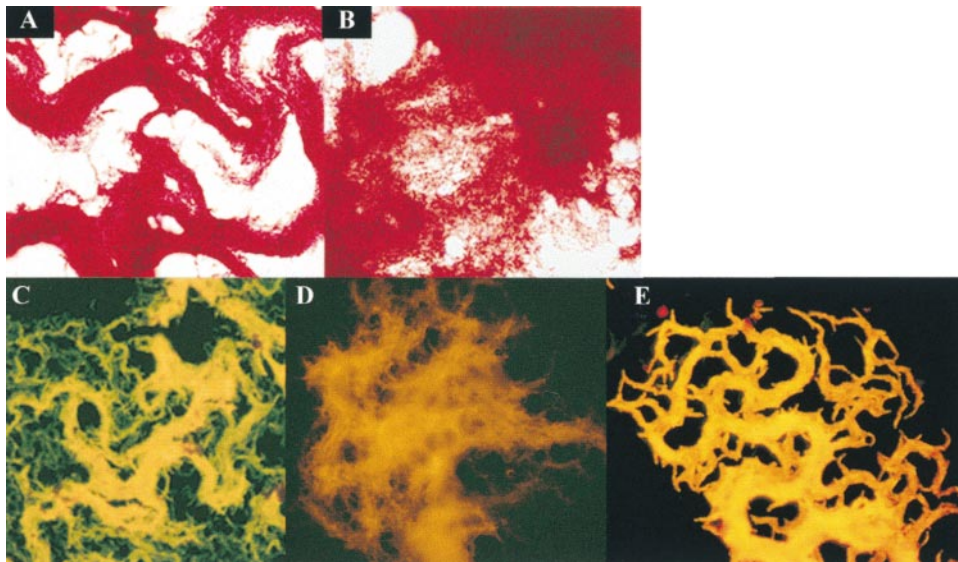


Figure 2. *pcaA* Mutants of BCG and *M. tuberculosis* Display Altered Microscopic Cording

All strains were grown and stained as described in Experimental Procedures. (A) and (B) are Ziehl-Neelsen stains at 600× magnification. (C)–(E) are Auramine-Rhodamine stains at 1000× magnification.

(A) BCG Pasteur *attB*::pYUB412 hygromycin.

(B) BCG Pasteur *pcaA*::Tn5370.

(C) *M. tuberculosis attB*::pMV306hyg.

(D) *M. tuberculosis ΔpcaA*::hyg.

(E) *M. tuberculosis ΔpcaA attB*::*pcaA*.

of four hygromycin-resistant clones contained the *pcaA* disruption.

We proceeded to analyze the cording phenotype of the *M. tuberculosis pcaA* mutant and found that *pcaA* contributes to cording in *M. tuberculosis* (Figures 2C

and 2D). The altered cording morphologies of both the *pcaA* mutants of BCG and *M. tuberculosis* strongly suggested that *pcaA* is involved in cording. Wild-type cording was restored when *pcaA* was reintroduced in single copy into the chromosome, confirming that the inactivation of *pcaA* is responsible for the noncording phenotype rather than the result of a polar effect on neighboring genes (Figures 1C and 1c; Figure 2E).

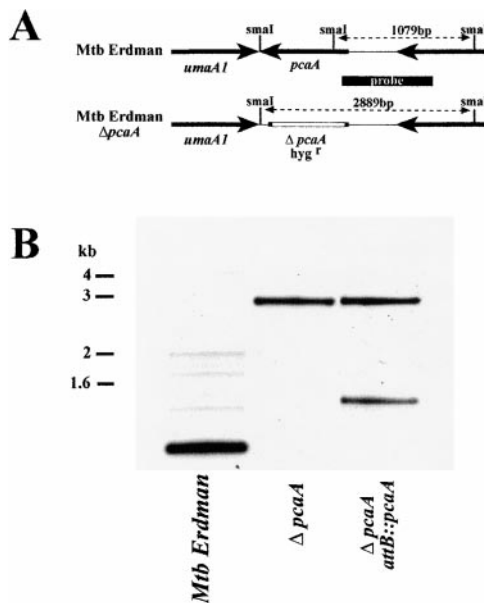


Figure 3. Disruption of *pcaA* in *M. tuberculosis* by Specialized Transduction

(A) Map of the *pcaA* genomic region in wild type and the *pcaA* mutant. Restriction sites and probe location are indicated.

(B) Southern blot of *Smal*-digested genomic DNA from indicated strains performed as described in Experimental Procedures.

pcaA Mutants Have Alterations in the Major Class of Mycolic Acids

Based on the strong sequence homology between *pcaA* and other cyclopropane synthases of mycobacteria, we reasoned that mutants in *pcaA* may lack specific cyclopropane rings in the mycolic acids of their cell walls. *M. tuberculosis* synthesizes three major classes of mycolic acids (Figure 4B). These three classes differ in the functional groups that are present on the meromycolate portion of the molecule. Alpha mycolates contain two *cis* cyclopropane rings, while both ketomycolates and methoxymycolates contain a *cis* or *trans* cyclopropane ring at the proximal position with their respective oxygen functions at the distal position. BCG Pasteur does not produce methoxymycolates (Minnikin et al., 1983, 1984) due to mutations in the *mma3* gene (Dubnau et al., 1998; Yuan et al., 1998).

To analyze the mycolic acid profiles of the *pcaA* mutants, we extracted total mycolic acids and analyzed them by conventional thin layer chromatography (TLC) and by argentation TLC. Silver nitrate retards the migration of unsaturated lipids relative to saturated or cyclopropanated lipids and has been used extensively to analyze cyclopropanation in mycolic acids (George et al., 1995; Yuan et al., 1995; Yuan and Barry, 1996). In the

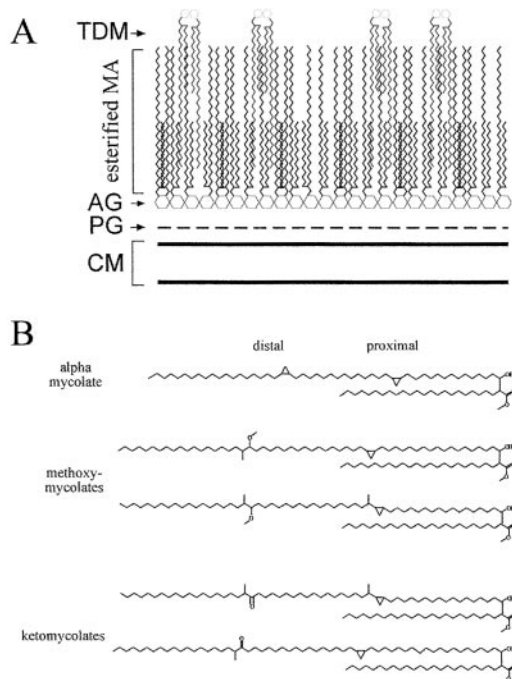


Figure 4. Mycolic Acids of *M. tuberculosis*: Cell Envelope Locations and Structures

(A) Schematic diagram of the cell envelope of *M. tuberculosis*. Exterior to the cell membrane (CM) and peptidoglycan (PG), mycolic acids are covalently linked to the arabinogalactan (AG), forming a thick hydrophobic layer. Mycolic acids in the form of trehalose dimycolate (TDM) are noncovalently associated with the cell envelope.

(B) Chemical structures of the major mycolic acids of *M. tuberculosis*. Methoxymycolates and ketomycolates have either *cis* or *trans* cyclopropane rings, while alpha mycolates contain only *cis* cyclopropanes.

absence of a cyclopropanating activity, mycolic acids would likely acquire an unsaturation in the meromycolate chain and therefore would be retarded by argentation TLC (Yuan et al., 1995). As shown in Figure 5A, the top migrating mycolic acid in the mutant strain that comigrates with wild-type alpha mycolate on conventional TLC is retarded on argentation TLC (Figure 5A, wild-type and $\Delta pcaA$ lanes of $AgNO_3$ side). In addition, the mycolic acid patterns of wild-type BCG are as described previously (Minnikin, 1982; Minnikin et al., 1984; Barry et al., 1998). In the *M. tuberculosis pcaA* mutant, three major mycolic acid species are visible but the relative ratios of alpha and ketomycolates are inverted such that ketomycolates are significantly more abundant in the mutant strain (Figures 5B–5D). As in the BCG *pcaA* mutant, inactivation of *pcaA* in *M. tuberculosis* alters the alpha mycolate such that it is retarded on argentation TLC (Figure 5B). Two-dimensional argentation TLC confirmed that the alpha mycolate in the *M. tuberculosis pcaA* mutant was retarded by silver and that these mutants contained no intact alpha mycolate (Figures 5C and 5D). Both the altered alpha mycolate and the overabundance of ketomycolate were present in the esterified and the TDM fractions of the cell wall (data not shown). Furthermore, we found a slight increase in the amount of *trans* cyclopropanated mycolic

acid in the *pcaA* mutant (20%) compared to wild type (12%, data not shown). For both BCG and *M. tuberculosis*, wild-type mycolate patterns were restored by complementation (Figures 5A and 5B, third and sixth lanes). The altered alpha mycolate was designated compound 1.

The CFAS in *E. coli* is expressed only as the organism enters stationary phase (Wang and Cronan, 1994). To examine whether the alteration of the alpha mycolate in the *pcaA* mutants was present in both exponential and stationary phase, we examined mycolic acids from logarithmic and stationary phase cultures of the BCG *pcaA* mutant. These experiments showed that the mycolic acid phenotype of the *pcaA* mutant is present in all phases of growth (data not shown). In addition, to examine whether PcaA protein expression is regulated by growth phase, we constructed an HA-tagged *pcaA* and showed that PcaA is present in logarithmic and stationary phase at equivalent levels (Figure 5E). These results demonstrate that *pcaA* is involved in synthesis of one or both of the two cyclopropane rings in the alpha mycolate molecule and, in contrast to CFAS, PcaA is not regulated by growth phase.

pcaA Synthesizes the Proximal Cyclopropane Ring of the Alpha Mycolates

To more specifically define the function of *pcaA* and its effect on cording, we investigated the chemical structure of compound 1. Compound 1 from the BCG *pcaA* mutant was purified by preparative TLC and analyzed by 600 MHz 1H NMR (Figure 6B). As has been previously shown for the alpha mycolate from wild-type *M. tuberculosis* (Barry et al., 1998), this spectrum demonstrates characteristic resonances of *cis* cyclopropane ring hydrogens at -0.34 and 0.64 . In contrast to alpha mycolate, and as predicted from its behavior on argentation TLC, compound 1 has a double bond, demonstrated by a multiplet at 5.33 ppm characteristic of vinyl protons. The coupling constant of these protons is 4.7 Hz, confirming a *cis* geometry of the double bond. Two-dimensional COSY NMR was performed and confirmed that the protons adjacent to the vinyl protons ($CH=CHCH_2$) have chemical shifts of 2.01, consistent with previously reported spectra (data not shown) (Yuan and Barry, 1996). As expected, there is no methyl branch in the meromycolate chain (no doublet at 0.95 ppm) (George et al., 1995). These one-dimensional and two-dimensional NMR data are consistent with a hybrid mycolic acid that contains a *cis* double bond and a *cis* cyclopropane ring without a methyl branch in the meromycolate chain, confirming that compound 1 is a derivative of the alpha mycolate of *M. tuberculosis*. The two likely possible structures for compound 1 are shown in Figure 6A (structures 2 and 3; intact alpha mycolate is structure 1 for comparison).

To determine the position of the unsaturation, the methyl ester of compound 1 from the *M. tuberculosis pcaA* mutant was oxidized with potassium permanganate and the cleavage products subjected to electrospray ionization mass spectroscopy (MS). The potential cleavage products of structures 2 and 3 are shown in Figure 6, structures 4 and 5. The mass spectrum of the oxidized lipids reveals a base peak of 621 atomic mass units (AMU), exactly the mass of the proximal fragment

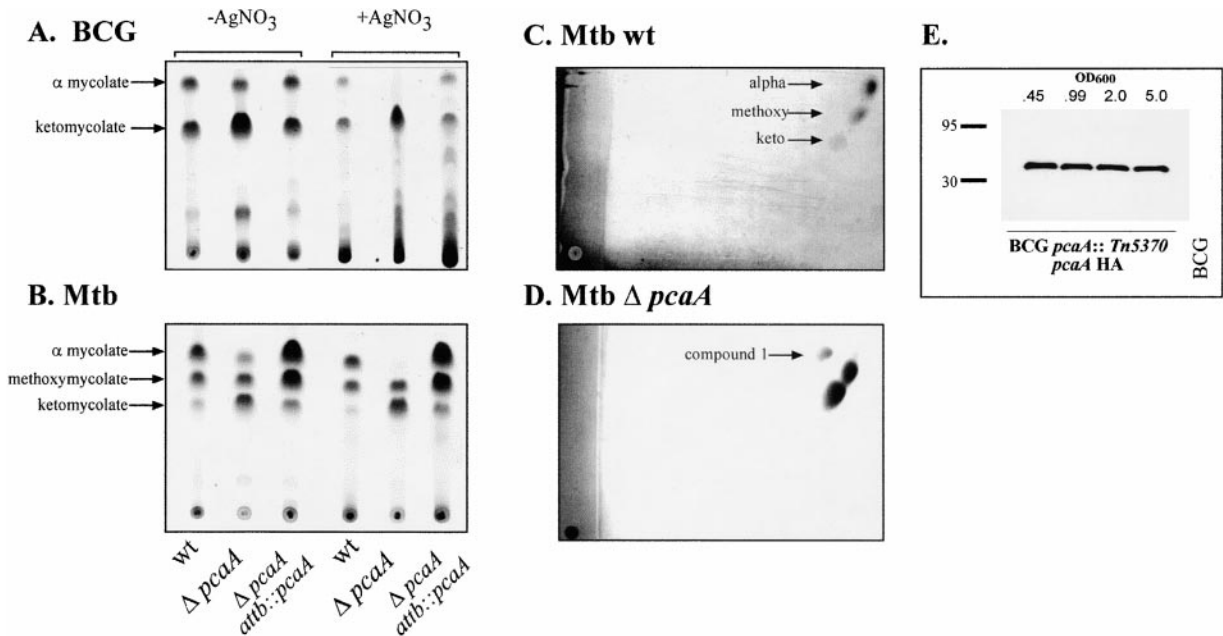


Figure 5. Inactivation of *pcaA* Affects Alpha Mycolate Synthesis in BCG and *M. tuberculosis*

(A and B) Mycolic acid methyl esters from the indicated strain were analyzed by thin layer chromatography as described in Experimental Procedures. The left panels are conventional silica, while the right panels are argentation TLC (abbreviated +AgNO₃).

(C and D) Two-dimensional argentation TLC of mycolic acid methyl esters from the indicated strain. In this TLC system, the plate is impregnated with silver nitrate except for a thin strip along the left edge. The first dimension of development is top to bottom along the unimpregnated strip and separates by polarity. The second dimension is left to right and achieves separation based on degree of unsaturation.

(E) PcaA is present in both logarithmic and stationary phase. The BCG *pcaA* mutant was complemented with an HA-tagged copy of PcaA on a multicopy plasmid, and cells were harvested at the indicated cell densities. Western blotting was performed with an anti-HA antibody.

of structure 4 having lost a methoxy group of 31 AMU (Figure 6C). The loss of a methoxy group from methyl esters of dibasic fatty acids during MS is common (Odham and Stenhagen, 1972). Negative ion mode MS produces ions that are 1 AMU lower than the parent compound, explaining the difference between the predicted mass of 622 and the observed mass of 621 on the negative ion mode spectra shown. The distal fragment of 549 AMU did not produce an ion. Importantly, no fragments consistent with cleavage at a distal double bond were observed (Figure 6, structure 5). Cleavage of compound 1 isolated from the BCG *pcaA* mutant was also consistent with cleavage at the proximal unsaturation (data not shown). These data show that in the absence of *pcaA*, a hybrid mycolate accumulates with a *cis* double bond at the proximal position in place of the *cis* cyclopropane present in wild-type alpha mycolate. Therefore, *pcaA* (proximal cyclopropane of alpha mycolates) is necessary for the synthesis of the proximal cyclopropane ring of the alpha mycolates.

pcaA Is Required for Long-Term Mycobacterial Persistence and Virulence

To test the relationship between cording, cyclopropane synthetases, and mycobacterial virulence, we intravenously infected C57Bl/6 mice with 10⁴ colony-forming units (CFU) of wild-type BCG and the BCG *pcaA* mutant. In this experiment, the wild-type organisms persisted indefinitely in the spleens of the mice, allowing long-term assessment of the survival of the strain in vivo

(Figure 7A) (Pelletier et al., 1982). Inactivation of *pcaA* did not affect initial growth of the organism over the first 3 weeks of the experimental infection. But after 6 weeks, when wild-type organisms persist at a constant level indefinitely, the *pcaA* mutant was progressively eliminated from the animal (Figure 7A). After 6 months of infection, the number of bacilli in the spleens of the animals infected with the *pcaA* mutant was 10-fold lower than wild type.

To confirm that this in vivo phenotype was applicable to fully virulent *M. tuberculosis*, we infected mice with the *M. tuberculosis* *pcaA* mutant. Surprisingly, the mutant strain replicated significantly more quickly than wild type over the first 3 weeks of the infection in all organs examined, reaching approximately 3-fold higher levels than wild-type organisms (Figures 7B–7D). The *M. tuberculosis* *pcaA* mutant was eliminated more quickly from the animal, such that colony counts 135 days after infection were significantly lower in the lung and liver despite the higher CFU at 3 weeks. The complemented strain behaved identically to wild type, demonstrating that both the initial enhanced replication and the persistence defect are secondary to inactivation of *pcaA*. The in vitro doubling time of all strains was the same (data not shown).

In addition, we observed a significant difference in mortality in the last group of animals infected with wild type and the *pcaA* mutant. By day 219 of the infection, 100% (5/5) of the animals infected with wild-type *M. tuberculosis* had died, while all of the animals infected

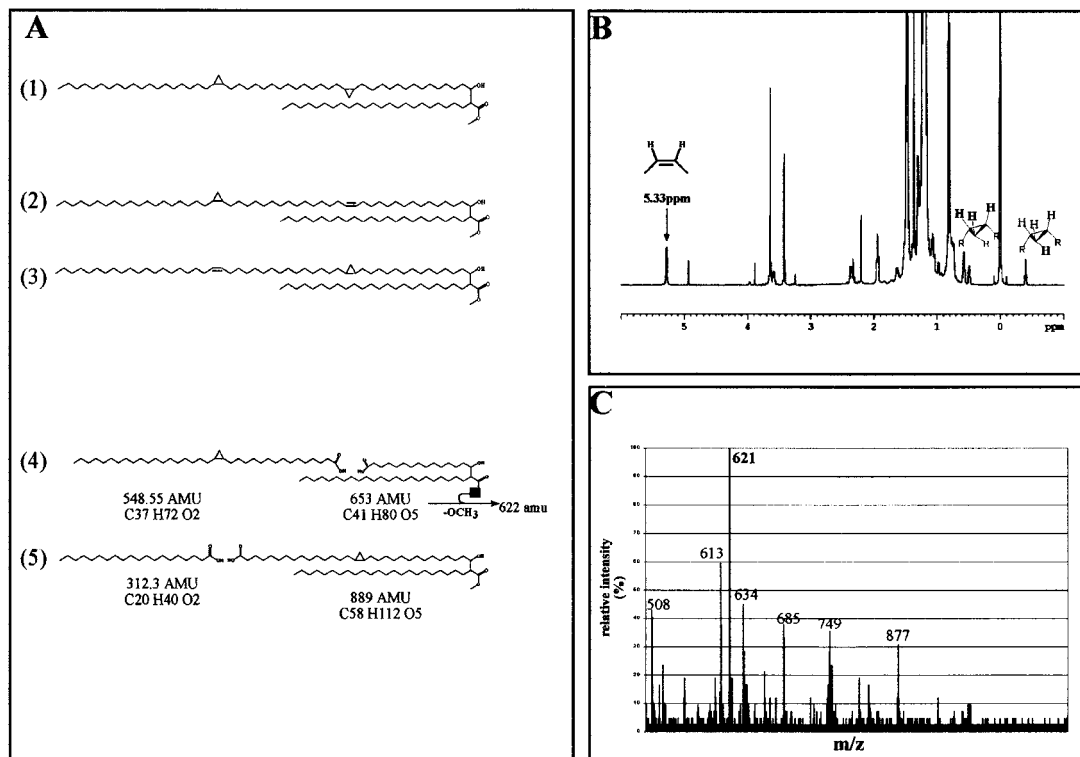


Figure 6. *pcaA* Is Required for Synthesis of the Proximal Cyclopropane Ring of the Alpha Mycolates

(A) Structures of relevant compounds for reference. Structure 1 is the alpha mycolate of *M. tuberculosis*. Structures 2 and 3 are two possible structures for compound 1 based on the TLC data presented in Figure 5 (see text) and the NMR data in (B). Structures 4 and 5 are the predicted cleavage products of structures 2 and 3, respectively, after permanganate oxidation. Molecular formulas and exact masses are given.

(B) Proton NMR of purified compound 1. The resonances of the *cis* cyclopropane and *cis* vinyl protons are labeled with structures. The coupling constant of the vinyl protons is 4.7 Hz, confirming a *cis* geometry. This spectrum is consistent with either structure 2 or structure 3 in (A).

(C) Electrospray ionization mass spectroscopy of permanganate cleaved compound 1. Compound 1 was oxidized with potassium permanganate, and the cleaved products were analyzed by mass spectroscopy. The predicted cleavage products are shown in structure lines 4 and 5 of (A). The mass spectrum demonstrates a base peak of 621 AMU consistent with the proximal fragment of structure 4 having lost a methoxy group (see text for explanation and arrow from structure 4 in [A]). The masses given in (A) are for the neutral charge state and are 1 AMU higher than the negative ion mode masses in (C).

with the *pcaA* mutant were alive and healthy (5/5), a highly significant difference in mortality (Figure 7E). Pathologic examination indicated that mortality differences were attributable to differences in severity of pulmonary damage. Lungs of wild-type infected mice showed extensive thickening of the interstitial septa by infiltration of macrophages and marked reduction in patent air spaces secondary to edema and histiocytic infiltration (Figure 7F). In contrast, the lungs of mice infected with the *pcaA* mutant showed large aggregations of lymphocytes localized around airways with extension of granulomatous inflammation only into the immediate surrounding parenchyma. The intervening alveolar spaces were patent without thickened septa (Figure 7G, arrow indicates typical lymphoid aggregate). Lungs from mice infected with the complemented mutant were pathologically identical to wild type. These results demonstrate that *pcaA* is required for long-term virulence of pathogenic mycobacteria in vivo and that inactivation of a single cyclopropane synthetase can dramatically alter the course of chronic infection with *M. tuberculosis*.

Discussion

In this study, we have examined the cording morphology of slow-growing mycobacteria and defined the function of a novel mycobacterial gene, *pcaA*, which is required for synthesis of the proximal cyclopropane ring of the alpha mycolic acids in both BCG and *M. tuberculosis*. In addition, we have shown that *pcaA* is required for the establishment of a lethal chronic *M. tuberculosis* infection. These results have important implications for our understanding of mycolic acid cyclopropane synthesis and define a role for cyclopropane synthetases in the virulence of pathogenic mycobacteria.

Implications for Understanding the Molecular Basis for Cording

Cording is a morphology of pathogenic mycobacteria that has been closely linked to virulence. However, the chemical entities in the mycobacterial cell envelope responsible for cording have remained unknown. A candidate compound, TDM, was named "cord factor" based

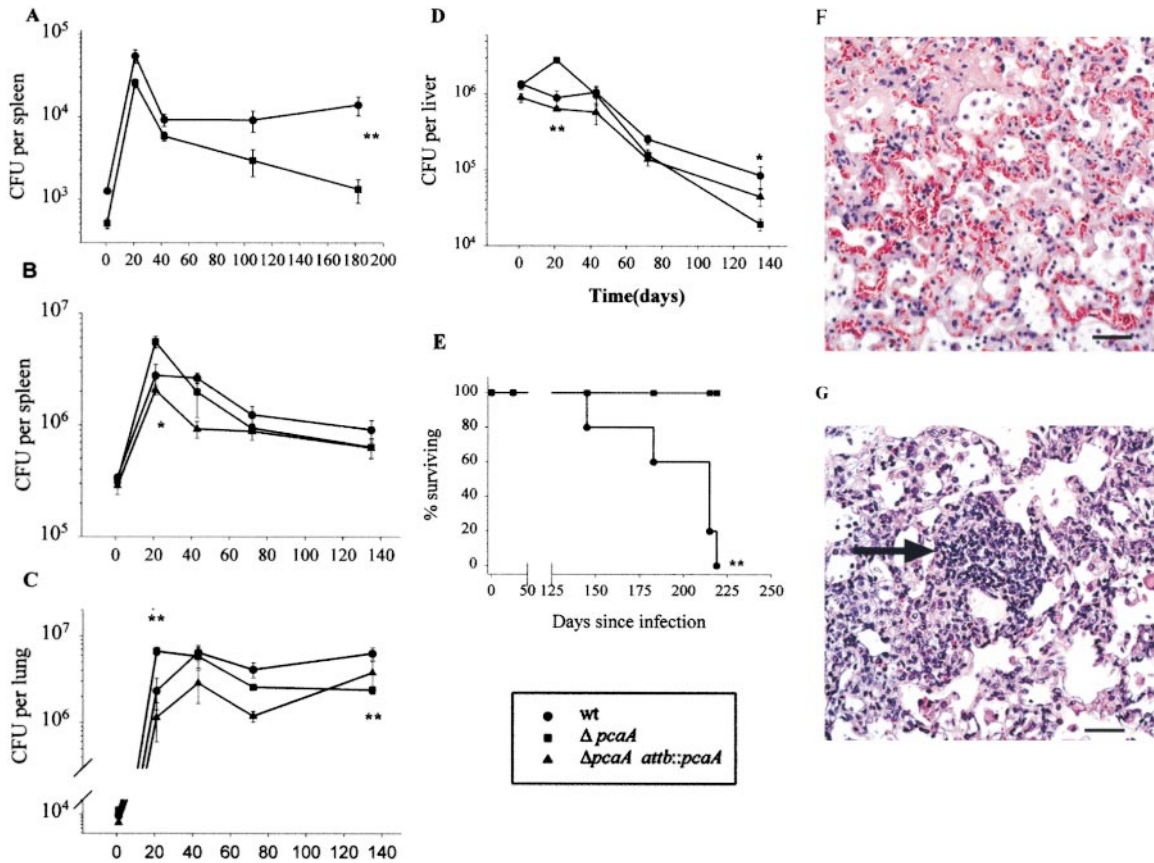


Figure 7. Loss of a Single Cyclopropane Synthase Affects Long-Term Persistence of BCG and *M. tuberculosis* In Vivo and Alters the Character of the Host Immune Response

(A) BCG *pcaA* mutant is eliminated from the spleens of mice. C57Bl6 mice were infected intravenously with 10^4 bacilli, and colony counts in the spleens were measured. Each time point represents the mean of four animals plotted on a log scale, and error bars are SEM.
 (B, C, and D) Inactivation of *pcaA* in *M. tuberculosis* enhances initial replication but confers a persistence defect. C57Bl6 mice infected with 10^6 *M. tuberculosis* intravenously. Colony counts in the spleen (B), lung (C), and liver (D) are plotted on a log scale. Each time point represents the mean of five animals except as noted in Experimental Procedures. Error bars are SEM.
 (E) *pcaA* is required for lethality *M. tuberculosis* infection. Five animals per group were followed until all wild-type infected animals had died, at which point the experiment was terminated.
 (F and G) Inactivation of *pcaA* alters the host inflammatory response. Hematoxylin and eosin-stained lung tissue from C57Bl6 mice infected with wild-type *M. tuberculosis* (F) demonstrates diffuse thickening of alveolar septa, pulmonary edema, histiocytic inflammation, and loss of patent air spaces. Lungs from animals infected with the *M. tuberculosis* *pcaA* mutant (G) demonstrate localized accumulations of lymphocytes (arrow), granulomatous inflammation in the adjacent parenchyma, and patent air spaces.
 * $p < 0.05$; ** $p \leq 0.01$ for comparison of wild type and $\Delta pcaA$.

on its presence in a petroleum ether extract of *M. tuberculosis* and its ability to inhibit the migration of leukocytes, a property that was known to be associated with the virulence of *M. tuberculosis* (Bloch, 1950). TDM consists of trehalose with mycolic acids esterified at the six positions of the sugars and is an impressively toxic glycolipid. TDM has been found in noncording mycobacteria, casting some doubt upon the role of these compounds in the cording phenotype. However, TDM is a class of molecules with a common structural motif but differing in fine chemical structure. Specifically, the mycolate side chains of TDM vary between mycobacterial species and reflect the mycolate composition of that species (Strain et al., 1977; Minnikin, 1982). Consequently, although TDM as a class of molecules is present in many nonpathogenic and noncording mycobacteria, the specific mycolic acid composition of *M. tuberculosis*

TDM is unique. These results raise the possibility that the cyclopropane content of TDM determines whether a strain forms cords, not whether or not it contains TDM. In addition, cording is likely to be a complex phenotype that can be affected by mutations affecting the synthesis of many cell envelope lipids.

Implications for Mycolate Biosynthesis

cmaA1 was the first mycobacterial gene shown to cyclopropanate mycolic acids (Yuan et al., 1995). This gene introduced a cyclopropane ring at the distal position of the α_2 mycolates of *M. smegmatis* when introduced on a multicopy plasmid. A second gene, *cmaA2*, exhibited a lack of substrate specificity in *M. smegmatis* as it cyclopropanated the proximal double bond of both the α_1 and the epoxy mycolates (George et al., 1995). A similar lack of substrate specificity was demonstrated

for the *mma2* gene, which cyclopropanated the proximal position of α_1 and epoxy mycolates when introduced into *M. smegmatis* (Yuan and Barry, 1996; Dubnau et al., 1997). The chromosomal location of *mma2* in an operon with other genes that appear to synthesize the methoxymycolates suggests that *mma2* synthesizes the proximal cis cyclopropane ring of the methoxy series. The results presented here demonstrate that *pcaA* is required for synthesis of the proximal cyclopropane of the alpha mycolates in both BCG and *M. tuberculosis*. We propose that either both *cmaA2* and *pcaA* are required for proximal cyclopropanation of the alpha series, or that *cmaA2* has another undefined role in mycolate cyclopropane synthesis. The large number of cyclopropane synthases in the genome of *M. tuberculosis* suggests that each member of this gene family may have a very specific nonoverlapping cyclopropane synthetic function. The presence of three distinct genes, *mma2*, *cmaA2*, and *pcaA*, each of which appears to introduce cis cyclopropane rings at the proximal position suggests that the substrate specificity of these enzymes is determined by the distal functional group present. This model predicts that the distal functional groups (methoxy, keto, and cyclopropane) would be added to the meromycolate chain before the proximal cyclopropane.

Cyclopropanation of Mycolic Acids in TB Pathogenesis

Our results demonstrate that a single cyclopropane synthase is necessary for the long-term persistence and virulence of pathogenic mycobacteria in vivo. Importantly, the in vivo phenotype of these *pcaA* mutants is specific for the persistence phase of the infection. The BCG mutant grows normally initially but is progressively eliminated from the animal after the onset of specific immunity at 3 weeks. The in vivo phenotype of the *M. tuberculosis pcaA* mutant was more complex than the BCG mutant. In contrast to BCG, inactivation of *pcaA* in *M. tuberculosis* enhanced growth over the first 3 weeks of the infection. Although the subsequent persistence defect is more subtle in *M. tuberculosis* than in BCG, the observed decrease in CFU is important because indefinite persistence in vivo is the hallmark of infection with *M. tuberculosis*, both in this mouse model and in humans. The observed difference between BCG and *M. tuberculosis* can be explained by the differences between the mycolic acid composition of the two mutant strains. Inactivation of *pcaA* in *M. tuberculosis* leads to a dramatic increase in the abundance of ketomycolates compared to wild-type *M. tuberculosis* (Figures 5C and 5D). Although ketomycolates are more abundant in the BCG *pcaA* mutant than in wild type, the relative increase is much less dramatic. Therefore, in contrast to BCG, the compensatory upregulation of ketomycolate synthesis in the *M. tuberculosis* mutant may compensate for the loss of intact alpha mycolate and modulate the drop in organism titer during the chronic phase of the infection (Yuan et al., 1998). In addition, the lack of methoxymycolates in BCG Pasteur may accentuate the effect of the *pcaA* mutation as a greater proportion of the cyclopropane residues (one of four) in the cell wall are lost compared with *M. tuberculosis* (one of six).

Despite the subtle effect of the *pcaA* mutation on

organism titers, inactivation of *pcaA* dramatically alters mortality from chronic *M. tuberculosis* infection. This nonlinear association between organism titer and mortality has been noted repeatedly in pathogenic mycobacteria but has not been explained mechanistically (Dunn and North, 1995; North et al., 1999). The pathologic differences observed between the wild-type and mutant infected animals suggest that alterations in mycolic acid subtypes may modulate the host immune response by influencing the composition of inflammatory cell infiltration. Further examination of the role of cyclopropanation in immune modulation will involve purification of the TDM from the *pcaA* mutant and testing its biologic activity in established assays of granuloma formation and TNF α release (Silva and Faccioli, 1988; Natsuhara et al., 1989; Behling et al., 1993). The in vivo phenotype observed with inactivation of a single cyclopropane synthetase among five such enzymes in the chromosome attests to the importance of these mycolate modifications for *M. tuberculosis* pathogenesis.

Although the in vivo phenotype of these mutants suggests a hypersusceptibility to the host immune response, the mechanism by which cyclopropane rings may be involved in persistence is not clear. Previous experiments have shown that distal cyclopropanation of the alpha mycolates of *M. smegmatis* increases the resistance of that strain to hydrogen peroxide (Yuan et al., 1995), although proximal cyclopropanation mediated by *cmaA2* did not affect hydrogen peroxide resistance (George et al., 1995). Inactivation of *pcaA* did not affect either hydrogen peroxide sensitivity or sensitivity to acidified sodium nitrite (Chan et al., 1992), a mycobacteriocidal nitric oxide donor (data not shown). At least as measured in vitro, loss of this proximal cyclopropane ring does not affect chemical sensitivity to the two major microbicidal effector mechanisms of human macrophages.

An attractive explanation for the relationship between cyclopropane rings and mycobacterial persistence is altered antigen presentation of mycolic acids by CD1. CD1 is an antigen-presenting molecule that presents lipid and glycolipid antigens including mycolic acids to T cells. The present model for lipid antigen presentation on CD1 suggests that the polar head groups of the lipids contact the T cell receptor and determine the specificity of antigen recognition while the lipid groups are buried in the hydrophobic pocket of CD1 (Kawano et al., 1997; Moody et al., 1997; Jackman et al., 1999). For a CD1-restricted T cell clone that responded to glucose monomycolate, variation in cyclopropane ring content of the mycolic acid did not affect T cell recognition (Moody et al., 1997). A similar analysis has not been published for a T cell line that recognizes free mycolic acids. In free mycolate, the carboxylic acid and the hydroxyl group (see Figure 4 for structures) are the only polar head groups available for TCR interaction. Under the model described above, a T lymphocyte responding to free mycolic acids presented on CD1 would respond equally well to any mycolic acid as these polar groups are invariant in mycolic acids. Future experiments will examine whether CD1-restricted T cell lines that recognize free mycolic acids respond equally to *M. tuberculosis* alpha mycolate and purified compound 1 from the *pcaA* mutant presented on CD1.

The majority of people infected with *M. tuberculosis* worldwide have latent infection and are an immense reservoir of people at risk for active TB. Although treatment of latent infection with isoniazid is effective for the prevention of active disease, therapy must be continued for at least 6 months (Anonymous, 1982) and can be associated with severe hepatic toxicity (Kopanoff et al., 1978). The identification of mycobacterial products that contribute to the long-term persistence of *M. tuberculosis* within the host will aid in the development of new treatments for this immense problem. Cyclopropane synthetases represent attractive drug targets as humans do not synthesize cyclopropanated fatty acids and drugs targeting this class of enzymes may shorten the duration of chemotherapy for TB. The advent of efficient allelic exchange in *M. tuberculosis* combined with the completion of the *M. tuberculosis* genome sequence will allow expansion of our understanding of the novel role of cyclopropane synthetases in *M. tuberculosis* pathogenesis.

Experimental Procedures

Bacterial Strains and Growth Conditions

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 (Boehringer Mannheim) at 50 µg/ml or Kanamycin (Sigma) at 20 µg/ml. BCG strains were grown similarly except albumin/dextrose/saline supplement was substituted for OADC. Cord reading agar was prepared as described (Lorian, 1966, 1969) with substitution of Triton X-100 0.0025% for Triton WR1339. Strain names are as follows: mc²2801, BCG Pasteur *pcaA::Tn5370*; mc²2802, mc²2801 (*attB::pcaA*); mc²3109, *M. tuberculosis* Erdman Δ *pcaA::hyg*; mc²3110, mc²3109 (*attB::pcaA*).

Screen for Noncording Mutants

BCG Pasteur was mutagenized using Tn5370 delivered by conditionally replicating mycobacteriophage as previously described (Bardarov et al., 1997) and plated on cord reading agar with Hygromycin. Individual colonies with altered colonial cording were picked into liquid media without Tween and examined for alterations in microscopic cording after auramine rhodamine staining (Difco). The site of transposon insertion was determined by digesting genomic DNA with XmaI and ligating into XmaI-digested pBluescript (Stratagene) and selecting for hygromycin. Sequence from the hygromycin cassette into surrounding genomic DNA was compared to the *M. tuberculosis* genome sequence.

Disruption of *pcaA* in *M. tuberculosis* by Specialized Transduction

A substrate for allelic exchange at *pcaA* was constructed by amplifying the flanking regions of *pcaA* by PCR from *M. tuberculosis* genomic DNA. Flanking regions of *pcaA* 908 bp 3' from the stop codon and 770 bp of 5' flank beginning at nucleotide 47 of *pcaA* were amplified, cloned with the TA cloning kit (Invitrogen), and sequenced. A hygromycin cassette flanked by resolvase sites was inserted between the two flanking regions to create pmsg72. A 3.5 kb XbaI-Asp718 fragment from pmsg72 was ligated to a 1.7 kb NheI-Asp718 fragment from pjsc90 to create pmsg78, a plasmid containing the knockout construct, a lambda cos site, and a unique PacI site. Details of the production of shuttle phasmids in mycobacteria and recombinant mycobacteriophages are contained in the references cited in this section. Briefly, PacI-digested pmsg78 was packaged into the unique PacI site of phae87, a temperature-sensitive mutant of the mycobacteriophage TM4, (Carriere et al., 1997) as previously described (Bardarov et al., 1997; Jacobs et al., 1987) and high titer pmsg78 prepared in *M. smegmatis* at 30°C. *M. tuberculosis* was grown to OD₆₀₀ 0.8, washed once with MP buffer (Jacobs et al., 1987), concentrated 10-fold in MP buffer, and infected

with pmsg78 at an MOI of 10 for 4 hr at 37°C. Cells were spun, resuspended in 7H9 media with 0.05% Tween 80, plated on hygromycin, and incubated at 37°C for 3 weeks. Hygromycin-resistant colonies were screened for allelic exchange by Southern blotting. Southern blotting was performed by alkaline transfer onto Hybond N⁺ nylon membranes (Amersham). Radioactive probes were prepared with Ready To Go DNA labeling beads (Pharmacia). For complementation, *pcaA* was amplified by PCR from genomic DNA with its putative promoter, cloned with the TA cloning kit, sequenced, and subcloned into pMV306kan, a site specific integrating mycobacterial vector.

Preparation and Analysis of Mycolic Acids

Mycolic acid methyl esters (MAME) were prepared from whole bacilli by incubation with 20% tetrabutylammonium hydroxide (Aldrich) at 100°C followed by methylation with methyl iodide and extraction with dichloromethane and drying under nitrogen. Crude MAMES were precipitated with toluene/acetonitrile (Besra, 1998). TLC was performed on Adsorbosil silica HPTLC plates (Alltech) with or without impregnation with 10% silver nitrate in water (George et al., 1995). Plates were developed by spraying with 20% sulfuric acid in ethanol and charring. For one-dimensional TLC, BCG samples were analyzed using three developments of hexanes:ethyl acetate 90:10, while TB mycolates were analyzed with hexanes:ethyl acetate 95:5. Two-dimensional TLC was performed as described previously with slight modification (George et al., 1995). The first dimension was four developments hexanes:ethyl acetate 95:5, and the second dimension was three developments hexanes:ethyl acetate 90:10. Preparative TLC was performed on 1000 µm silica preparative uniplates (Analtch). Silica was extracted with ether, filtered through 0.45 µm PVDF syringe filters (Whatman), and MAMES precipitated with toluene/acetonitrile. For permanganate oxidation, mycolate was dissolved in 5:1 hexanes:acetic acid and incubated with 0.28 M KMnO₄ at 0°C with violent stirring for 4 hr. The reaction was quenched with saturated sodium sulfite, and the products were recovered by acidification and extraction with chloroform.

Construction of HA-Tagged PcaA and Analysis of PcaA Expression

The *pcaA* gene, including 500 bp of promoter sequences yet missing the termination codon, was amplified by PCR and inserted into pJSC77 to create pJSC264. This placed the HA epitope (YPYDVP-DYA) at the C terminus of the protein, and the entire fusion gene was confirmed by sequencing. SDS-PAGE and Western blotting was performed on whole-cell extracts from cultures harvested at the indicated densities according to standard protocols with an anti-HA-11 monoclonal antibody (Berkeley Antibody Company).

NMR and MS Analysis

Proton NMR was performed on a Bruker DRX600 spectrometer in deuteriochloroform. Mass spectra of permanganate cleaved compound 1 was performed on either a PE-SCIEX API-III triple-quadrupole mass spectrometer or a Finnigan LCQ ion-trap mass spectrometer in negative ion mode. The sample was infused into the mass spectrometer at 2–5 l/min using a Harvard Apparatus syringe pump after diluting with dichloromethane/chloroform/methanol (1/1/1).

Animal Infections

C57Bl/6 mice (Jackson labs) were infected by lateral tail vein injection. Organs were harvested at appropriate time points, and homogenized and serial dilutions were plated for determination of colony-forming units. For BCG animal infections, four animals per time point were used. For *M. tuberculosis* animal infections, five animals per time point were used except for the 72 day mc²3109 time point where four animals were used secondary to contamination of the organ homogenates in one animal and the 135 day time point of mc²3110 where four animals were harvested. Pathologic examination was performed on tissues fixed in 10% buffered formalin. Statistical comparisons were made using the Student's t test.

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