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Phosphorylation of Mycobacterial PcaA Inhibits Mycolic Acid Cyclopropanation

CONSEQUENCES FOR INTRACELLULAR SURVIVAL AND FOR PHAGOSOME MATURATION BLOCK[§]

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Background: Mycolic acid cyclopropanation plays an important role in mycobacterial virulence but molecular mechanisms controlling cyclopropanation remain unknown.

Results: Phosphorylation of the cyclopropane synthase PcaA decreases mycolic acid cyclopropanation and intracellular survival and abrogates the phagosome maturation block (PMB).

Conclusion: PMB modulation is dependent on mycobacterial protein phosphorylation.

Significance: This study highlights Ser/Thr-dependent phosphorylation of a mycolic acid biosynthetic enzyme in mycobacterial virulence.

Pathogenic mycobacteria survive within macrophages by residing in phagosomes, which they prevent from maturing and fusing with lysosomes. Although several bacterial components were seen to modulate phagosome processing, the molecular regulatory mechanisms taking part in this process remain elusive. We investigated whether the phagosome maturation block (PMB) could be modulated by signaling through Ser/Thr phosphorylation. Here, we demonstrated that mycolic acid cyclopropane synthase PcaA, but not MmaA2, was phosphorylated by mycobacterial Ser/Thr kinases at Thr-168 and Thr-183 both *in vitro* and in mycobacteria. Phosphorylation of PcaA was associated with a significant decrease in the methyltransferase activity, in agreement with the strategic structural localization of these two phosphoacceptors. Using a BCG $\Delta pcaA$ mutant, we showed that PcaA was required for intracellular survival and prevention of phagosome maturation in human monocyte-derived macrophages. The physiological relevance of PcaA phosphorylation was further assessed by generating PcaA phosphoablative (T168A/T183A) or phosphomimetic (T168D/T183D) mutants. In contrast to the wild-type and phosphoablative *pcaA* alleles, introduction of the phosphomimetic *pcaA* allele in the $\Delta pcaA$ mutant failed to restore the parental mycolic acid profile and cording morphotype. Importantly, the PcaA phosphomi-

metic strain, as the $\Delta pcaA$ mutant, exhibited reduced survival in human macrophages and was unable to prevent phagosome maturation. Our results add new insight into the importance of mycolic acid cyclopropane rings in the PMB and provide the first evidence of a Ser/Thr kinase-dependent mechanism for modulating mycolic acid composition and PMB.

Tuberculosis (TB)³ caused by *Mycobacterium tuberculosis* (*Mtb*), remains a major threat to global health, claiming the life of two million individuals annually (1). *Mtb* is an obligate human pathogen predominantly growing within host phagocytes. In these cells, bacilli reside in phagosomes, which they prevent from undergoing maturation and fusion with lysosomes (2, 3). In this manner, the bacilli remain in a weakly acidic and non-cytolytic environment. An early requirement for the phagosome maturation block (PMB) is the establishment of an all-around close apposition between the phagosome membrane and the mycobacterial surface that will occur only when phagosomes contain a single mycobacterium (loner phagosomes) (reviewed in Ref. 4). Whenever a phagosome contains more than one mycobacterium or mycobacterial clumps (social phagosome), the close apposition is not maintained in regions spanning adjacent bacteria. Such phagosomes systematically mature and fuse with lysosomes (reviewed in Ref. 4). Likewise, physical abrogation of the close apposition, by binding of par-

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³ The abbreviations used are: TB, tuberculosis; CFU, colony-forming unit; EM, electron microscopy; FAS-II, type II fatty acid synthase; HMDM, human monocyte-derived macrophage; *Mtb*, *Mycobacterium tuberculosis*; PMB, phagosome maturation block; SAH, S-adenosyl-homocysteine; SAM, S-adenosyl-methionine; STPK, Ser/Thr protein kinase; TDM, trehalose dimycolate; TLC, thin layer chromatography.

Regulation of Mycolic Acid Cyclopropanation

ticles to the mycobacterial surface (3), leads to phagosome maturation and fusion with lysosomes. Mycobacteria are, however, not destroyed in phagolysosomes. Instead, they can rescue themselves from this cytolytic environment to again reside in non-maturing phagosomes that no longer fuse with lysosomes (Ref. 3, reviewed in Ref. 4). This, however, does not make the PMB redundant. The preferred site of residence for a mycobacterium in host macrophages remains a non-matured phagosome, regardless of whether this is achieved directly by the PMB or by re-establishing it after rescue from phagolysosomes (3). In addition, the PMB could be a strategy for sequestering pathogenic mycobacteria away from antigen-presenting compartments, thereby altering the host immune response (5).

The molecular basis for the PMB is the focus of intense research. Several mycobacterial determinants, including cell surface-associated lipids such as lipoarabinomannan (LAM) and cord factor, as well as proteins, were reported to take part in this process (6–9). Among these, the mycobacterial Ser/Thr Protein kinase (STPK), PknG, secreted into macrophage phagosomes, is one of the candidates mediating both the PMB and intracellular survival of BCG and *Mtb* (10). It has, therefore, been proposed that modulation of phagosome-lysosome fusion by PknG could be mediated through phosphorylation of host proteins following secretion within the macrophage (10).

The family of STPK, expressed by both BCG and *Mtb*, is composed of 11 members (11). Whether membrane-associated STPKs play a role in regulating phagosome maturation through phosphorylation of mycobacterial proteins remains to be established. Recent studies have demonstrated the participation of STPKs in the regulation of many physiological pathways, such as those connected to cell shape/division and cell envelope biosynthesis (12). In particular, it was shown that all major type II fatty acid synthase (FAS-II) components are phosphorylated by STPKs and that, in general, phosphorylation inhibits the activity of the FAS-II enzymes (12–16). These studies culminated with the recent demonstration that InhA, the primary target of isoniazid, is controlled via phosphorylation and that phosphorylation of InhA severely impeded enoyl reductase activity, mycolic acid biosynthesis, and mycobacterial growth in a manner similar to isoniazid (17). Although these studies suggest that *Mtb* controls in a very subtle manner its FAS-II system by regulating each step of the elongation cycle, no information is available regarding whether STPK-dependent phosphorylation may also regulate the activity of enzymes involved in functional modification of mycolic acids, such as cyclopropane synthases. Previous studies identified the *pcaA* gene as required for mycolic acid cyclopropanation in both BCG and *Mtb* (18). The corresponding *pcaA* mutants failed to produce serpentine cords and, importantly, were unable to persist within and kill infected mice, indicating that PcaA is necessary for the establishment of a lethal and chronic infection (18). An attractive hypothesis is that regulation of PcaA activity by phosphorylation represents a strategy employed by pathogenic mycobacteria to regulate mycolic acid cyclopropanation and, as a consequence, mycobacterial pathogenesis.

Herein, we explored whether PcaA represents an endogenous substrate for mycobacterial STPKs and whether it participates in the PMB and intracellular survival of bacilli in human

monocyte-derived macrophages. From our data, we propose that STPK-dependent phosphorylation of PcaA plays a critical role in regulating mycolic acid cyclopropanation, which in turn affects phagosome processing and intramacrophage survival.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Growth Conditions—*Escherichia coli* strains used for cloning and expression of recombinant proteins are listed in supplemental Table S1. Bacteria were grown in LB medium at 37 °C. Media were supplemented with ampicillin (100 µg/ml) or spectinomycin (100 µg/ml) as required. BCG Pasteur 1173P2 and mc²2801 (BCG Pasteur *pcaA*::Tn5370) (18) strains were grown on Middlebrook 7H10 agar plates with OADC enrichment (Difco) or in Sauton's medium containing 0.05% tyloxapol (Sigma) with kanamycin (25 µg/ml) or hygromycin (50 µg/ml) when required.

Cloning, Expression, and Purification of PcaA, MmaA2, and Mutant Proteins—*pcaA* and *mmaA2* were amplified by PCR using *Mtb* H37Rv chromosomal DNA as a template and primers listed in supplemental Table S2. Amplified products were cloned into the pETPhos vector (19), generating pETPhos_ *PcaA* and pETPhos_ *MmaA2*. Site-directed mutagenesis was directly performed on pETPhos_ *PcaA* using inverse PCR amplification with self-complementary primers (supplemental Table S2). The duet strategy (20) was used to generate phosphorylated PcaA in the pCDFDuet-1 vector harboring the PknF kinase domain. All constructs were verified by DNA sequencing. The different His-tagged recombinant proteins were overexpressed in *E. coli* C41(DE3) and purified using TALON Metal affinity resin (Clontech) as described previously (14). Cloning, expression, and purification of GST-tagged STPKs were described earlier (16).

Overexpression and Purification of PcaA from BCG—*pcaA* was cloned into the shuttle vector pVV16 (21) using the primers listed in supplemental Table S2. The resulting construct pVV16_ *PcaA*_ *WT* was used for directed mutagenesis to generate the pVV16_ *PcaA*_ *T168A/T183A* mutant. The constructs were electroporated into mc²2801. Transformants were grown and used for purification of the His-tagged PcaA protein as described above. The recombinant proteins were used for immunoblotting using anti-phosphothreonine antibody according to manufacturer's instructions (Santa Cruz Biotechnology).

In Vitro Kinase Assay—*In vitro* phosphorylation was performed as described (22) with 4 µg of PcaA or MmaA2 in 20 µl of buffer P (25 mM Tris-HCl, pH 7.0; 1 mM DTT; 5 mM MgCl₂; 1 mM EDTA; 50 µM cold ATP) with 200 µCi/ml (65 nM) [γ -³³P]ATP (PerkinElmer, 3000 Ci/mmol) and 0.6–4.2 µg of kinase to define the optimal autophosphorylation activity of each kinase for 30 min at 37 °C.

Enzymatic Assays—Methyltransferase activity of PcaA and MmaA2 was measured by using the couple assay for mycolic acids as described (23) with slight modifications. Briefly, reactions were performed in a total volume of 600 µl and followed spectrophotometrically at 412 nm to detect the conversion of SAH (methyltransferase product) to homocysteine by the *Mtb* SAH hydrolase. Assays were performed in presence of 100 mM (pH 7.5) phosphate buffer containing 1.5 µM SahH, 250 µM

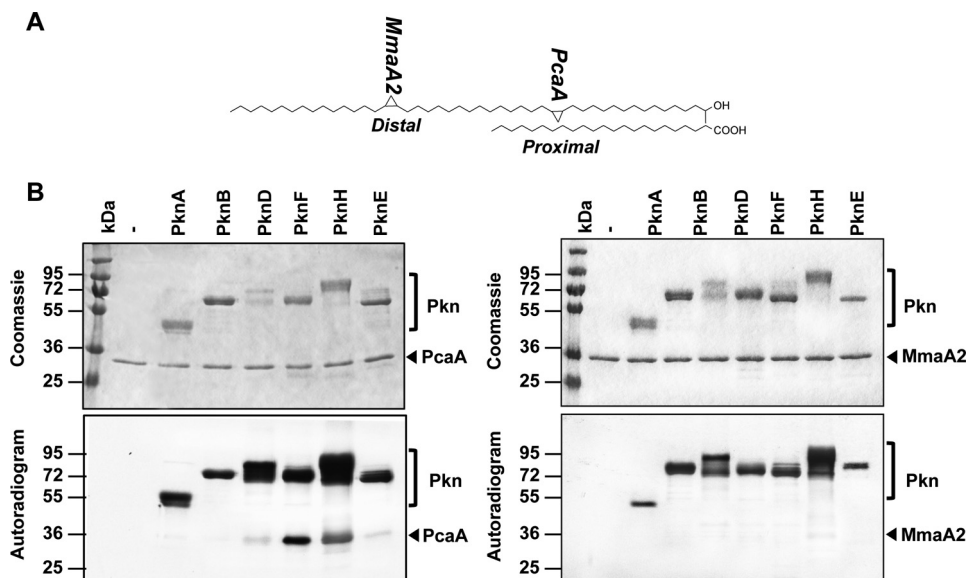


FIGURE 1. *Mtb* PcaA is phosphorylated by multiple STPKs. A, structure of *Mtb* α -mycolic acids. Sites of cyclopropanation by PcaA and MmaA2 are indicated. B, *in vitro* phosphorylation of PcaA (left panels) and MmaA2 (right panels) by multiple kinases. *Mtb* STPKs purified as GST fusions were incubated with His-tagged PcaA or MmaA2 and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Samples were separated by SDS-PAGE, stained with Coomassie Blue or visualized by autoradiography. Upper bands reflect the autophosphorylation activity of each kinase (Pkn); the lower bands correspond to phosphorylated PcaA.

NAD, and $40\ \mu\text{M}$ of unsaturated fatty acids (*cis*, *cis*-11,14-eicosadienoic acid or linoleic acid, Sigma) pre-equilibrated for 5 min at 37° . After addition of $400\ \mu\text{M}$ 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), the solution was blanked and added to pre-incubated $100\ \mu\text{M}$ SAM and $2\ \mu\text{M}$ of either PcaA or MmaA2 to start the reaction.

Complementation Studies and Immunoblotting—*mc*²2801 was transformed with pMV261, pMV261_PcaA_WT, pMV261_PcaA_T168A/T183A or pMV261_PcaA_T168D/T183D (supplemental Table S1) constructed as follows. The *pcaA* gene (lacking the stop codon) and a 500-bp promoter sequence was cloned in fusion with an HA epitope in the C-terminal position by using the primers listed in supplemental Table S2. Site-directed mutagenesis was directly performed on pMV261_PcaA_WT using inverse PCR amplification with self-complementary primers (supplemental Table S2). Western blotting was performed on whole-cell extracts from cultures harvested at early stationary phase according to standard protocols with an anti HA-11 (12CA5) monoclonal antibody (Roche).

Analysis of the Mycolic Acid Profile—Mycobacterial cultures were metabolically radiolabeled with $1\ \mu\text{Ci/ml}$ [$2\text{-}^{14}\text{C}$]acetate ($56\ \text{mCi/mmol}$, Amersham Biosciences) and added to mid logarithmic phase cultures for 5 h. Labeled mycolic acids were then extracted as described previously (24) and loaded onto thin layer chromatography (TLC) silica-coated plates. Mycolic acids were then separated using petroleum ether/acetone (95/5, v/v) and exposed overnight to a film.

Cording Assay—Approximately 50 colony-forming units (CFU) were plated on cord reading agar, incubated at 37°C for 3 weeks and observed under a light microscope MVX10 (Olympus).

Purification of Blood Monocytes, Differentiation into Macrophages (HMDM), and Infection—Human blood samples, purchased from the French National Blood provider of Montpel-

lier, were collected from fully anonymized non-tuberculous control donors. This study was conducted according to the principles expressed in the Helsinki Declaration. Purified monocytes, isolated as described (25), were seeded onto 24-well plates at a density of $7 \times 10^5/\text{ml}$ in complete culture medium (RPMI containing 10% FCS) and differentiated into macrophages (HMDM) with rh-M-CSF ($10\ \text{ng/ml}$) for 7 days. HMDM were infected with exponentially growing BCG cultures ($\text{DO} = 0.8$) at an MOI of 1:1 or 5:1. Single-cell bacterial suspensions were generated by $2 \times 10\text{s}$ pulses in a water-bath sonicator, followed by passage through a 26-gauge needle to disrupt remaining bacterial clumps. Before infection, residual bacterial aggregates were removed by low-speed centrifugation ($120 \times g$) for 2 min. Infected cells were washed with PBS and reincubated in bacteria-free medium. For CFU scoring, cells were lysed with 0.1% Triton X-100 in PBS at selected times post-infection. Serial dilutions of the lysate were plated onto Middlebrook 7H10 agar medium supplemented with OADC. Colonies were counted after incubation at 37°C for about 3 weeks.

Labeling of Endomembrane Compartments with Endocytic Tracers—Two methods were used as follows: (i) horseradish peroxidase (HRP) uptake. At 1 or 5 days post-infection, infected cells were incubated for 90 min at 37°C in complete medium containing $25\ \mu\text{g/ml}$ HRP (26). Endocytosis was stopped by fixing the cells overnight with 2.5% glutaraldehyde prepared in 0.1 M cacodylate buffer, pH 7.2, containing 0.1 M sucrose, 5 mM CaCl_2 and 5 mM MgCl_2 . Cells were then processed for HRP cytochemistry as described below (26). (ii) Latex bead uptake and transfer to lysosomes. Uninfected cells were washed twice with medium and exposed for 30 min at 37°C to hydrazide-modified latex beads of $0.1\ \mu\text{m}$ in diameter diluted in medium. After three washes in medium, cells were incubated for 2 h at 37°C in bead-free medium to chase the tracer to lysosomes (27). Cells were then infected with BCG at a MOI of 5:1 for 4 h,

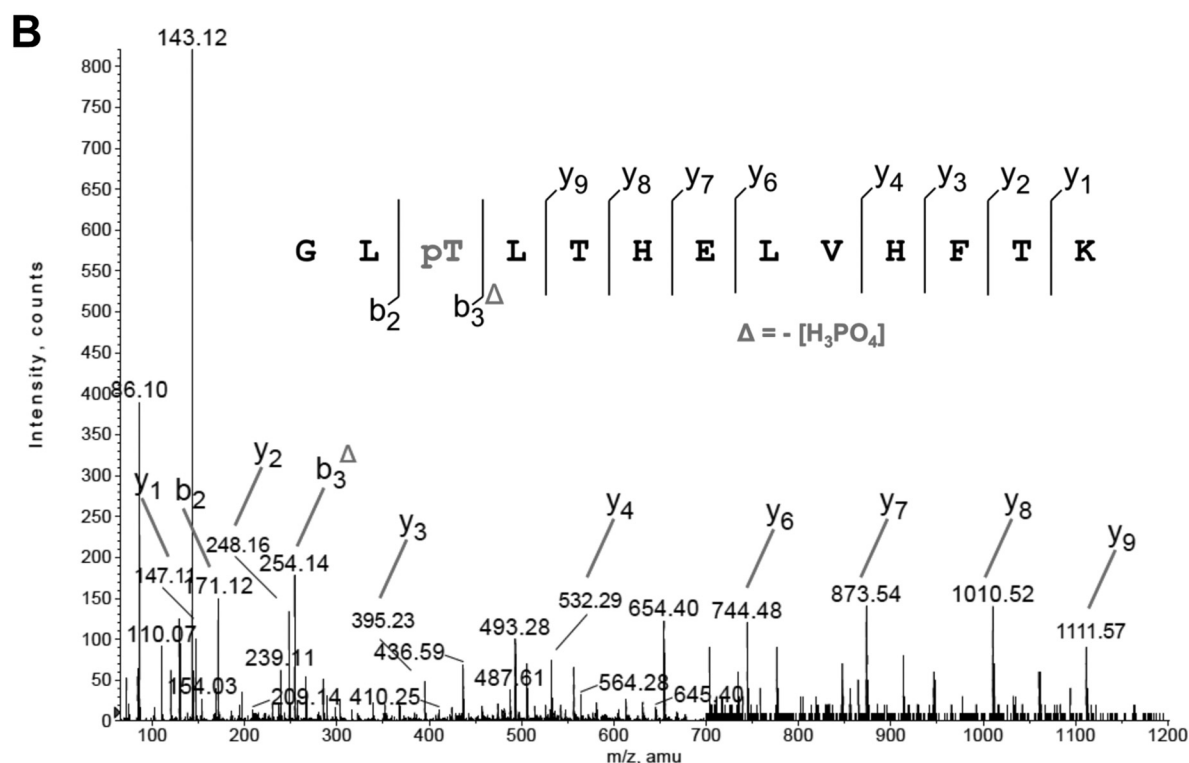
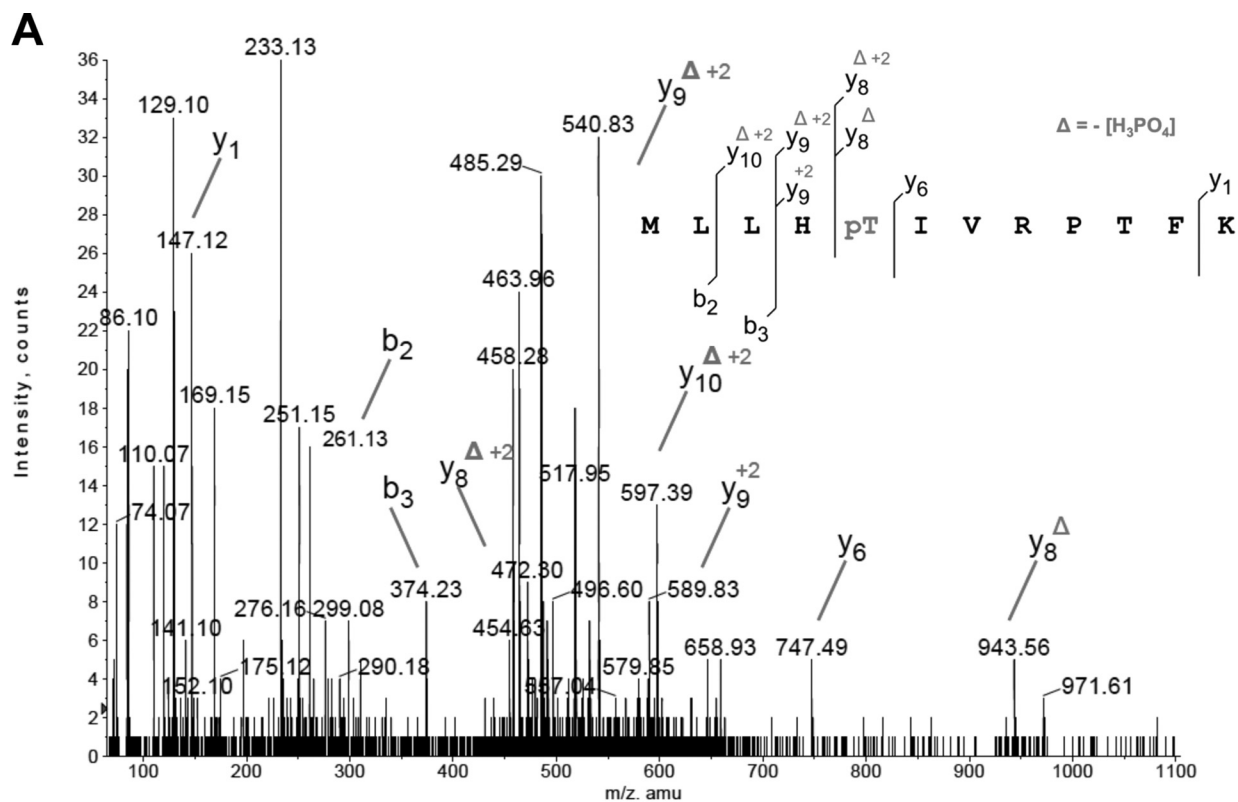


FIGURE 2. **Identification of the PcaA phosphorylation sites.** A, MS/MS spectra at m/z 517.95 (+3) of peptide (164–175) of PcaA. Unambiguous location of the phosphate group on Thr-168 was shown by observation of the “y” C-terminal daughter ion series. Starting from the C-terminal residue, all “y” ions lose phosphoric acid (–98 Da) after the phosphorylated residues. B, MS/MS spectra at m/z 525.94 (+3) of peptide (181–193) of PcaA. Unambiguous location of the phosphate group on Thr-183 was shown by observation of the “y” C-terminal daughter ion series. Starting from the C-terminal residue, all “y” ions lose phosphoric acid (–98 Da) after the phosphorylated residues.

washed, and re-incubated in BCG-free medium. One day later, cells were fixed with glutaraldehyde and processed for EM.

Horseradish Peroxidase Cytochemistry—Cells exposed to HRP and fixed with glutaraldehyde were washed overnight at 4 °C with sucrose-containing buffer. After three washes with sucrose-free buffer, cells were incubated with 3,3'-diaminobenzidine tetrahydrochlorate (DAB)-H₂O₂ as before (26) and then processed for EM.

Processing for Transmission Electron Microscopy—Cells fixed with glutaraldehyde were processed as described (28). Briefly, cells were washed with complete cacodylate buffer, and post-fixed for 1 h at room temperature with 1% osmium tetroxide in the same buffer devoid of sucrose. They were washed with buffer, scraped off the dishes, concentrated in 2% agar in cacodylate buffer and treated for 1 h at room temperature with 1% uranyl acetate in Veronal buffer. Samples were dehydrated in a graded series of ethanol and embedded in Spurr resin. Thin sections (70 nm-thick) were stained with 1% uranyl acetate in distilled water and then with lead citrate and observed under the electron microscope (Zeiss 912).

Quantitation of Loner Phagosomes with Lysosomal Material—In all cases, 50–100 different loner phagosomes taken at random from three different experiments were examined for the presence or absence of lysosomal material. Care was taken to avoid serial sections.

RESULTS

PcaA Is Phosphorylated in Vitro by Multiple Ser/Thr Kinases—We first sought out to determine whether PcaA and MmaA2, that introduce cyclopropanes at the proximal and distal positions on the α -meromycolic acid, respectively (18, 29) (Fig. 1A) were modified by phosphorylation. This changes the physicochemical properties of defined Ser or Thr residues by introducing negative charges that ultimately affect protein activity. The kinase domains of several *Mtb* STPKs (PknA to PknL), expressed as GST-tagged fusion proteins (16), were incubated with purified *Mtb* PcaA or MmaA2 and [γ -³³P]ATP. SDS-PAGE/autoradiography analysis indicated that PcaA, but not MmaA2, was phosphorylated by several kinases (Fig. 1B). Based on the intensity of the radioactive signal corresponding to phosphorylated PcaA, PknF appears to be the most efficient kinase to phosphorylate PcaA *in vitro*. Signals were weak with PknD, PknE, and PknH that all display various autokinase activities (16) but none were detected with PknA or PknB. Although MmaA2 is structurally highly related to PcaA (30), only PcaA is a specific substrate and interacts with PknF (Fig. 1B). This suggests that cyclopropanation of the α -mycolic acid proximal position may be regulated in mycobacteria by extracellular cues.

PcaA Is Phosphorylated on Thr-168 and Thr-183—Mass spectrometry (MS) was then used to identify the number and nature of phosphorylation sites on PcaA, as reported previously for other STPK substrates (13, 14, 17, 31, 32). Phosphorylated PcaA was purified from *E. coli* co-expressing PknF and PcaA (pETDuet-*pcaA*) and subjected to MS analysis after tryptic digestion. Sequence coverage of 94% was obtained, bearing all possible Ser and Thr residues. Phosphorylation occurred only on peptides (164–175) (Fig. 2A) and (181–193) (Fig. 2B) with

phosphate groups on Thr-168 and Thr-183. To prevent phosphorylation, Thr-168 and Thr-183 were substituted by alanine, either individually or together. The corresponding PcaA_T168A, PcaA_T183A, and PcaA_T168A/T183A proteins were expressed and purified as His-tagged proteins in *E. coli* BL21(DE3)Star harboring either pETPhos-*pcaA_T168A*, pETPhos-*pcaA_T183A*, or pETPhos-*pcaA_T168A/T183A*. Following incubation with PknF and [γ -³³P]-ATP, SDS-PAGE/autoradiography revealed that single Ala point mutations were associated with a strong radioactive signal decrease (Fig. 3A). Total abrogation of phosphorylation occurred in the T168A/T183A double mutant compared with PcaA_WT (Fig. 3A). Similar results were obtained when the mutant was incubated with other STPKs (Fig. 3B), thus identifying PcaA_T168A/T183A as a PcaA phosphoablative mutant.

To address the relevance of PcaA phosphorylation in mycobacteria, recombinant BCG overexpressing PcaA_WT was analyzed by Western blotting using anti-phosphothreonine antibodies. Antibody specificity for the phosphorylated isoform was first assessed using PcaA purified from either *E. coli* (pET-Phos-*pcaA*) or *E. coli* co-expressing PknF and PcaA (pETDuet-*pcaA*). Phosphorylated PcaA from pETDuet-*pcaA*, but not unphosphorylated PcaA from pETPhos-*pcaA*, was specifically recognized by the antibodies (Fig. 3C). That PcaA from BCG carrying pVV16-*pcaA* was in a phosphorylated state was confirmed by a specific band recognized by the anti-phosphothreonine antibodies (Fig. 3C). Western blot analysis of recombinant PcaA purified from either exponential or stationary cultures of BCG carrying pVV16-*pcaA* showed similar levels of PcaA phosphorylation. This suggests that PcaA phosphorylation is growth-phase independent (Fig. 3C). No specific phosphorylation signals were detected in PcaA_T168A/T183A purified from BCG carrying pVV16-*pcaA_T168A/T183A*, thus excluding the existence of additional phosphorylation sites. Therefore, phosphorylation occurs at Thr-168 and Thr-183 both *in vitro* and in mycobacteria.

Phosphorylation Decreases PcaA Methyltransferase Activity—Multiple sequence alignments of PcaA orthologues from various mycobacterial species indicated that both Thr-168 and Thr-183 are conserved in *Mtb*, *M. marinum*, and *M. leprae*, whereas the second phosphorylation site (corresponding to Thr-183 in the *Mtb* sequence) is substituted by a proline in the *M. smegmatis* orthologue (Fig. 4A). Phosphorylation site mapping on the three-dimensional structure (30) emphasized the strategic roles of both threonine residues on PcaA activity (Fig. 4B). Thr-168 is located within the β -sheet of the core SAM-methyltransferase fold with the threonine side-chain facing a mobile protein segment that refolds upon cofactor-binding (33). Thr-183 was located in the so-called α 2- α 3 specific motif of mycolic acid SAM-methyltransferases seemingly involved in the interaction with the mycobacterial acyl carrier protein AcpM (30, 33). This motif, which delineates the entrance of the hydrophobic channel from the enzyme surface to the active site, displays the largest deviation when comparing the known structures of mycolic acid SAM-methyltransferases (33). The above data suggest that Thr-168 and Thr-183 are the unique phosphoacceptors in PcaA that directly influence its enzymatic

Regulation of Mycolic Acid Cyclopropanation

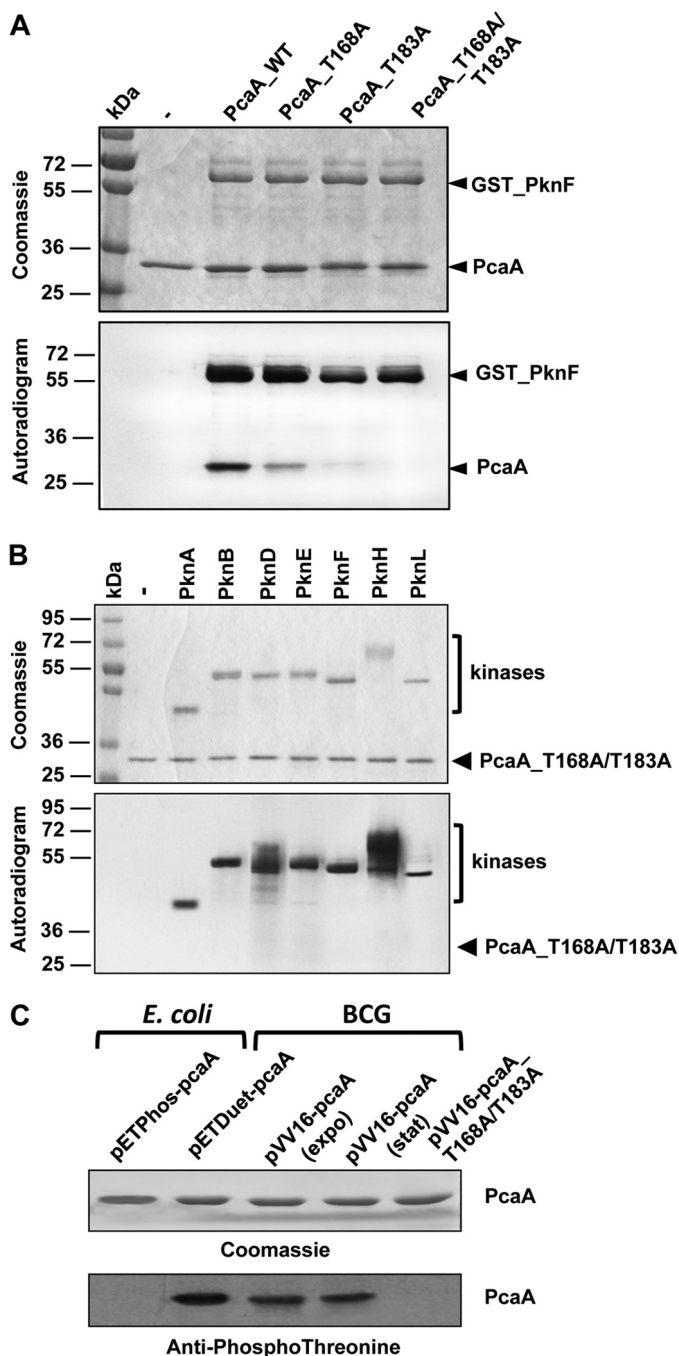


FIGURE 3. Phosphorylation of PcaA variants with Ala-substituted phospho-sites. A, purified PcaA_{WT}, PcaA_{T168A}, PcaA_{T183A}, and PcaA_{T168A/T183A} mutants were incubated with PknF and [γ -³³P]ATP, separated by SDS-PAGE, stained with Coomassie Blue, or visualized by autoradiography. B, *in vitro* phosphorylation of PcaA_{T168A/T183A} by multiple kinases. Recombinant STPKs encoded by the *Mtb* genome were expressed and purified as GST fusions and incubated with purified His-tagged PcaA_{T168A/T183A} and radiolabeled [γ -³³P]ATP. Depending on the STPK, 0.6 to 4.2 μ g were necessary for obtaining optimal autophosphorylation activity for each specific kinase. Samples were separated by SDS-PAGE, stained with Coomassie Blue (upper panels) and visualized by autoradiography after overnight exposure to a film (lower panels). C, phosphorylation of PcaA in mycobacteria. *E. coli* harboring pETPhos-PcaA was used as a source of non phosphorylated PcaA; the strain harboring pETDuet-PcaA coexpressing PknF and PcaA provides the phosphorylated PcaA isoform. PcaA_{WT} or PcaA_{T168A/T183A} were produced in exponentially growing or stationary recombinant BCG strains. 3 μ g of purified His-tagged PcaA variants were probed with anti-phosphothreonine antibodies.

activity. This prompted us to compare the methyltransferase activity of phosphorylated and non-phosphorylated isoforms using a coupled assay originally developed for CmaA2 (23). This colorimetric assay is based on the detection of *S*-adenosylhomocysteine (SAH) conversion to homocysteine by SAH hydrolase. Two unsaturated fatty acids were used as substrates of *Mtb* PcaA and MmaA2; *cis*, *cis* 11,14-eicosadienoic acid and linoelaidic acid (Fig. 4C). Although these lipids are structurally distinct from the authentic unsaturated meromycolyl-AcpM substrates, both PcaA and MmaA2 catalyzed cyclopropanation of double bonds in presence of either lipid (Fig. 4, D and E). This reaction was inhibited by dioctylamine as reported earlier for CmaA2 (23) (data not shown). Importantly, the activity of phosphorylated PcaA, derived from *E. coli* carrying pETDuet-PcaA, was significantly reduced by 40 to 50% compared with non-phosphorylated PcaA from *E. coli* carrying pETPhos-PcaA, irrespective of the lipid substrate. Conversely, the MmaA2 methyltransferase activity remained unchanged whether or not it was produced from the pETPhos or pETDuet vectors, consistent with the fact that MmaA2 is not phosphorylated by STPKs (Fig. 1B). These results indicate that, *in vitro*, both PcaA and MmaA2 catalyze the introduction of cyclopropane rings on *cis*, *cis* 11,14-eicosadienoic acid and linoelaidic acid and support the critical role of STPK-dependent phosphorylation in regulating PcaA activity.

BCG PcaA Phosphomimetic Mutants Display Altered Phenotypes—Acidic residues such as aspartic acid (Asp) qualitatively mimic the phosphorylation effect with regard to functional activity (13–15, 17, 34). Therefore, phosphoablative (Thr to Ala replacements) and phosphomimetic (Thr to Asp replacements) *pcaA* alleles were generated and introduced into BCG mc²2801 (BCG Pasteur *pcaA*::Tn5370) (18). In these constructs, WT, phosphoablative (T168A/T183A) and phosphomimetic (T168D/T183D) PcaA isoforms were fused to a HA tag and their expression was monitored by Western blotting using anti-HA antibodies. As expected, BCG Δ *pcaA* transformed with the empty vector (pMV261) failed to express PcaA whereas similar amounts of PcaA_{WT}, PcaA_{T168A/T183A} and PcaA_{T168D/T183D} were synthesized in the corresponding strains (Fig. 5A), allowing subsequent phenotype comparison. Whether phosphorylation of PcaA affects *de novo* mycolic acid biosynthesis was next investigated by labeling the various BCG strains with [1-¹⁴C]acetate followed by mycolic acid extraction and thin layer chromatography (TLC)/autoradiography analysis. BCG mc²2801 exhibited an altered α -mycolic acid profile (18, 35), that could be restored by complementation with either WT or phosphoablative PcaA (Fig. 5B). In contrast, introduction of PcaA_{T168D/T183D} failed to restore the parental mycolic acid profile (Fig. 5B and supplemental Fig. S1) indicating that the phosphomimetic isoform is unable to complement the lack of PcaA activity in mc²2801, presumably as a consequence of its reduced methyltransferase activity (Fig. 4D). This effect was not due to a tertiary structure change, as suggested by modeling of the PcaA_{T168D/T183D} mutant structure. Indeed, introduction of Asp at position 168, inaccessible to solvent, and at position 183, that is solvent exposed, does not seem to induce steric or electrostatic conflicts that could alter the protein fold (supplemental Fig. S2). Thus, it can be inferred

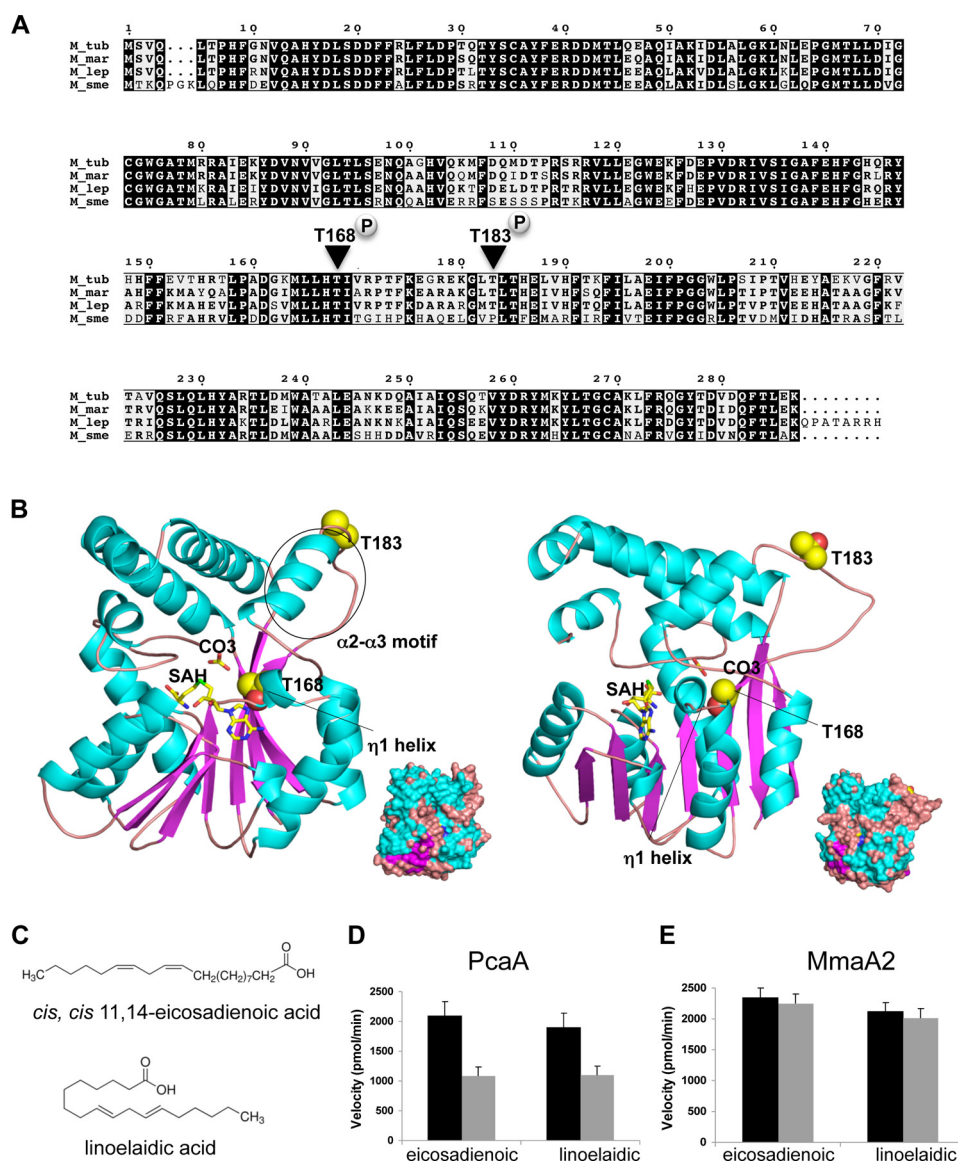


FIGURE 4. Phosphorylation of PcaA decreases methyltransferase activity. *A*, conservation of the phosphoacceptors in PcaA orthologues. The multiple sequence alignment of PcaA orthologues was performed using ClustalW and Esript (M_tub, *Mycobacterium tuberculosis*; M_mar, *Mycobacterium marinum*; M_lep, *Mycobacterium leprae*; M_sme, *Mycobacterium smegmatis*). Numbering of the amino acids corresponds to the PcaA protein from *Mtb*. Residues conserved in all species are presented in black boxes. The phosphorylated site of PcaA from *Mtb* and their positions are indicated. *B*, localization of Thr-168 and Thr-183 phospho-sites in the three-dimensional structure of PcaA. Two perpendicular views of the PcaA structure (30); PDB entry 1L1E) are shown in ribbon representation with α -helices in cyan, β -strands in magenta, and turns and coils in salmon. SAH, the cofactor product, and the carbonate ion found in the active site are displayed as sticks with carbons in yellow, nitrogens in blue, oxygens in red, and sulfur in green. The two threonine side chains are shown as spheres with the same color code. For each view, insets represent the protein molecular surface. Structure visualization and analyses were carried out using the PyMOL program. *C*, structures of *cis, cis*-11,14-eicosadienoic acid or linoelaidic acid, an all-*trans* fatty acid homolog of linoleic acid. *D*, PcaA-dependent methyltransferase assays. Activity of the non-phosphorylated and phosphorylated PcaA isoforms were assayed and compared using the two different lipids. Activity of the non-phosphorylated protein (pETPhos-PcaA, black bars) and activity of phosphorylated PcaA (pETDuet-PcaA) are shown in gray. Values are means \pm S.E. of triplicates representative of three sets of experiments with independent protein preparations. *E*, a similar approach was used to determine the MmaA2 activity.

that PcaA phosphorylation in mycobacteria reduces/abolishes its activity, supporting the view that this post-translational modification plays a key role in regulating α -mycolic acid cyclopropanation.

The effect of constitutive PcaA phosphorylation on the colonial morphology of BCG was next examined. Although no differences were observed for the different BCG strains cultured in liquid medium (Fig. 6B), mc²2801 grew far more slowly than its parental strain on agar plates, as evidenced by colony size (supplemental Fig. S3). This growth defect could be fully restored by complementing the mutant strain with either

PcaA_WT or PcaA_T168A/T183A, but not with the phosphomimetic mutant. Cording morphology, in which bacteria are intertwined into serpentine rope-like structures, is a distinctive feature of pathogenic mycobacteria. The characteristic mc²2801 cord formation defect (18) was restored following complementation with either WT or phosphoablative *pcaA* alleles. However, the phosphomimetic construct failed to restore cord formation (Fig. 5C). Thus, PcaA phosphorylation is strongly associated with a defective mycolic acid profile, subsequently affecting mycobacterial growth and serpentine cord formation.

Regulation of Mycolic Acid Cyclopropanation

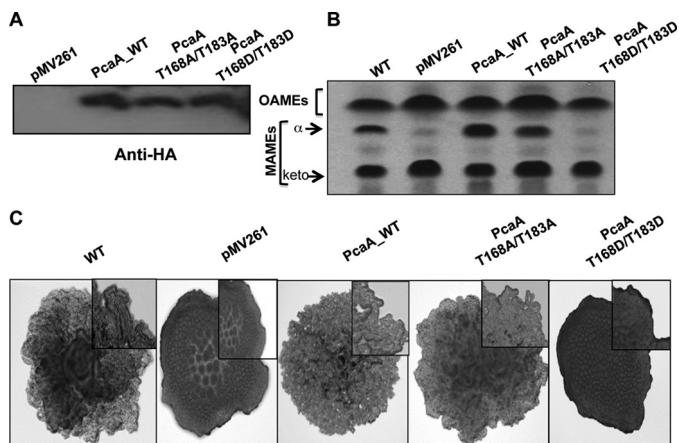


FIGURE 5. Phosphorylation of PcaA is associated with an altered mycolic acid profile and loss of cording phenotype. *A*, overexpression of the various PcaA variants in BCG $\Delta pcaA$. BCG mc²2801 was transformed with pMV261, pMV261_pcaA_WT, pMV261_pcaA_T168A/T183A, and pMV261_pcaA_T168D/T183D constructs for constitutive expression of the various pcaA alleles fused to a C-terminal HA epitope. 40 μ g of crude lysates were subjected to immunoblotting and probed with anti-HA antibodies. *B*, mycolic acid profile of the various BCG transformants. Equal counts (50 000 cpm) of labeled lipids were loaded and analyzed by TLC/autoradiography using petroleum ether/acetone (95/5, v/v). Positions of α - and keto-mycolic acid methyl ester are indicated. OAME, oleic acid methyl ester. *C*, cording morphology of BCG strains. Single BCG colonies were grown on cord-reading agar and visualized after 3 weeks. Magnification, $\times 100$.

Functional PcaA Is Required for Intracellular Survival and PMB—Although PcaA cyclopropanation is required for *Mtb* persistence and pathology in late stages of infection in mice (18), its role during early infection remains unclear. This prompted us to investigate the ability of BCG $\Delta pcaA$ to infect and replicate within human monocyte-derived macrophages (HMDM). Determination of intramacrophagic bacterial counts showed that survival of the mutant strain was severely compromised as compared with the WT strain (Fig. 6A). This was not due to an inherent growth defect as the mutant and parental strains replicated equally well in liquid medium (Fig. 6B).

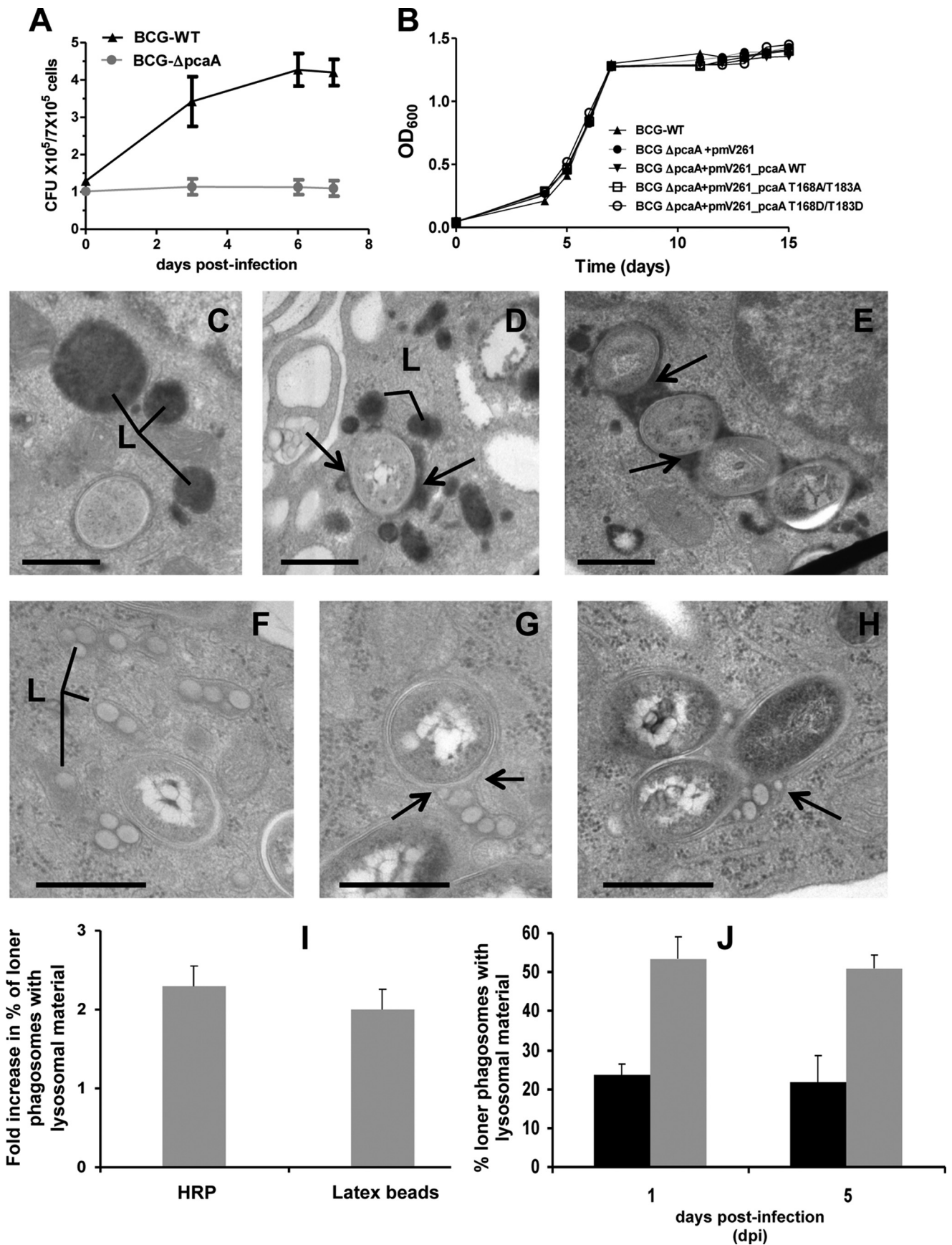
Pathogenic mycobacteria survive in macrophages by residing in phagosomes which they prevent from maturing and fusing with lysosomes (2, 36). We sought out to examine the putative role of PcaA in this process. Cells were exposed to electron-dense endocytic tracers by two different approaches. In the first, small latex beads were chased to lysosomes prior to phagocytic uptake of the WT or the $\Delta pcaA$ strains; in the second, the endocytic tracer horseradish peroxidase (HRP) was added at selected time points after infection (1 or 5 days post-infection) with the above strains and the cells were stained for HRP by EM cytochemical methods. Phagosome processing was analyzed in terms of fusion and intermingling of contents between phagosomes and the different endomembrane compartments, namely early endosomes (eEN) and late endosomes/lysosomes (indicated as Ly in text and figures) that can be easily distinguished from one another by the presence of latex beads (only in Ly) and the cytochemical staining pattern for HRP (37). To analyze phagosome processing, it is important to distinguish between loner and social phagosomes since, as shown before, the PMB does not hold for social phagosomes that invariably mature and fuse with lysosomes (reviewed in Ref. 4). Therefore, when looking for the effects of PcaA on the PMB,

observations and quantitations had to be limited strictly to loner phagosomes. One must also keep in mind that non-matured phagosomes fuse with eEN but are unable to fuse with Ly (4). With both strains, two types of loner phagosomes were encountered, *i.e.* (i) immature phagosomes either displaying a thin rim of HRP reaction product as is typical of eEN (not shown) or no marker (Fig. 6, C and F), and (ii) phagosomes that contain lysosomal material indicating that they have matured and fused with Ly (Fig. 6, D and G). As before, social phagosomes, containing two or more mycobacteria, all contained lysosomal material (Fig. 6, E and H). A quantitative analysis showed a 2-fold increase in the percentage of loner phagosomes displaying lysosomal material for BCG $\Delta pcaA$ -containing phagosomes as compared with the WT strain (Fig. 6I). A more complete study done with HRP as endocytic tracer showed that the percentage of phagolysosomes remained stationary for at least 5 days post-infection (50% for the BCG $\Delta pcaA$ mutant *versus* about 25% for the WT strain) (Fig. 6J). Overall, these results emphasize the major contribution of PcaA in the PMB.

Phosphorylation of PcaA Inhibits Intramacrophage Growth and Affects Phagosome Maturation—The above data indicate that phosphorylation of PcaA is linked to a defective cell wall-associated mycolic acid profile in BCG and affects its intracellular growth. We next determined the consequences of PcaA phosphorylation on both intramacrophage BCG growth (Fig. 7A) and the PMB (Fig. 7B). For both studies, HMDM were infected with the parental BCG strain, the $\Delta pcaA$ mutant or the different BCG complemented strains. Over a 6-day period the CFU count increased 4–5-fold for the parental strain and for both BCG $\Delta pcaA$ strains complemented with the WT or phosphoablative isoforms. The phosphomimetic strain was unable to replicate within HMDM, similarly to the $\Delta pcaA$ mutant (Fig. 7A). Concerning the PMB, only 30% of the loner phagosomes containing the $\Delta pcaA$ mutant complemented with the WT or phosphoablative PcaA contained lysosomal material, similarly to the parental WT strain. In contrast, 60% of the loner phagosomes containing the phosphomimetic strain contained lysosomal material, as it was the case for the $\Delta pcaA$ mutant (Fig. 7B). Altogether, these results indicate that phosphorylation of PcaA prevents growth and abrogates the PMB.

DISCUSSION

The present study extends previous work showing the regulatory role of phosphorylation on mycolic acid chain length and functionalization during its biosynthesis (12). More importantly, it provides the first evidence for a Ser/Thr kinase phosphorylation-dependent regulation of cyclopropane synthase. This mechanism is of special relevance as cyclopropanes are major contributors to the physiopathology of *Mtb* infection (18). Phosphorylation of Thr-168 and Thr-183 residues caused a strong decrease in methyltransferase activity *in vitro*. In the case of growing bacteria, a phosphomimetic mutant displayed a major alteration of the mycolic acid profile due to the lack of di-cyclopropanation of α -mycolic acids, similar to what is observed for the $\Delta pcaA$ mutant. Likewise, impaired cyclopropanation was associated with altered colony morphology and inability to form serpentine cords (Fig. 8). Major phenotypical alterations have also been observed in *in vivo* situations. Inac-



Regulation of Mycolic Acid Cyclopropanation

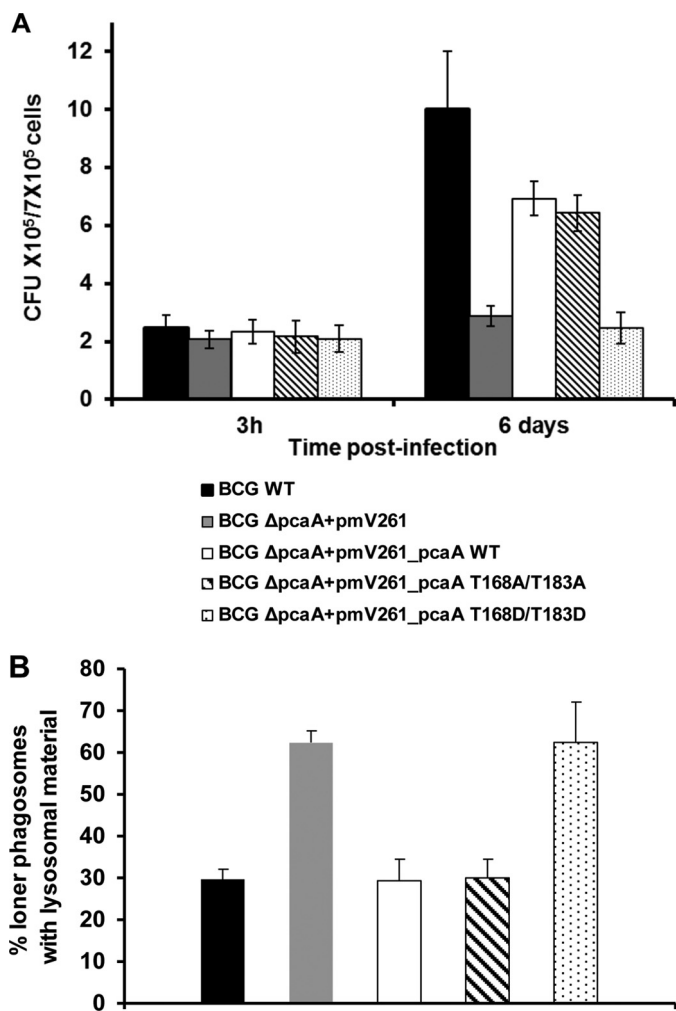


FIGURE 7. Phosphorylation of PcaA inhibits intracellular growth and abrogates PMB. HMDM were infected with different BCG strains at an MOI of 1:1 (A) or 5:1 (B). A, intracellular CFU counts enumerated at 3 h and day 6 post-infection. B, percentage of loner phagosomes containing lysosomal material after exposure to the endocytic tracer HRP added to infected cells for 90 min at day 1 post-infection. Both experiments (A, B) correspond to the means \pm S.E. (n = samples from three different donors).

tivation of *Mtb* *pcaA* causes attenuation of virulence and a less severe granulomatous pathology in mice (18). In addition, PcaA-dependent cyclopropanation of TDM, a direct effector of *Mtb* pathogenesis, regulates its inflammatory activity (18, 38). In particular, TDM purified from a PcaA mutant is hypoinflammatory for macrophages during early stages of infection (38).

We show here that phosphorylation of PcaA not only arrests intracellular mycobacterial replication but also abrogates the PMB. In addition to the requirement for a tight apposition of the phagosome membrane to the mycobacterial surface all around (3, 4, 39), which suggests an intimate cross-talk between the mycobacterium and its host cell, a large number of both

cellular and mycobacterial molecules were seen to play a role in, or correlate with, the PMB and intracellular survival of mycobacteria (4, 40, 41). This leads to the question of whether any one of these is sufficient by itself and, if so, why is there such redundancy (3)? Present knowledge rests upon two types of experimental approaches: either eliminating the function of the molecular factor concerned and/or reconstituting the factor in an artificial system. With these approaches, *i.e.* deletion of *pcaA* and construction of phosphomimetic or phosphoablative mutants, we have studied phagosome maturation for a sufficiently long time (5 days *versus* a few hours only in many other studies) to conclude that mycolic acid cyclopropanation by PcaA maintains the PMB and that PcaA phosphorylation, by inhibiting cyclopropanation, abrogates this block indefinitely. It is noteworthy that part of the phagosomes, however, do not mature and fuse with lysosomes upon PcaA phosphorylation, suggesting that other mycobacterial factors such as TDM (9, 42) or phospho-signaling proteins like PknG (10), the protein tyrosine phosphatase PtpA (43, 44) and the secreted lipid phosphatase SapM (45) might also be important mycobacterial factors. Among these, only SapM and PtpA seem to interfere directly with host physiological processes leading to prevention of phagosome maturation, whereas PknG seems to affect phagosome maturation through phosphorylation of yet unknown host proteins following its secretion within macrophage phagosomes.

Previous genetic screens identified several *Mtb* mutants defective in PMB (8, 46) and affected in various pathways, including lipid synthesis. Of notable interest is the *Mtb* *pcaA::Tn* mutant that failed to arrest phagosome maturation and trafficked to late phagosomal compartments in bone-marrow derived macrophages (46), thus supporting the view that the data obtained with BCG in the present study are relevant to *Mtb*.

With the PMB being the most conspicuous survival mechanism of pathogenic mycobacteria, why does *Mtb* phosphorylate PcaA? One must keep in mind that the fate of pathogenic mycobacteria does not rely exclusively on its own defense mechanisms. Host cells have developed several strategies for combating invading pathogens which, in turn, have developed several strategies for using the host environment to their advantage. In the present case, an attractive hypothesis would be that PcaA phosphorylation is driven by cell host factors located in the phagosome membrane and hence in direct contact with the mycobacterial surface due to the close apposition between the two structures. These factors would act as a signal for triggering autophosphorylation of mycobacterial STPKs, inducing in turn PcaA phosphorylation (Fig. 8). Mycolic acid structural alteration could affect the close apposition between the phagosome membrane and the mycobacterial surface. As a result, phagosomes would mature and fuse with lysosomes, as observed in the present study. However, whether PcaA phos-

FIGURE 6. PcaA requirement for intracellular survival and arrest of phagosome maturation. A, replication of BCG WT and BCG Δ pcaA in HMDM infected at a MOI of 1:1. CFU were scored at the indicated time points. Results are representative of three independent experiments from three different blood donors. B, growth curves of different BCG strains in broth medium. C–H, acquisition of endocytic tracer (C–E, HRP; F–H, latex beads) by BCG-WT- or BCG Δ pcaA-containing phagosomes. HMDM were infected with BCG WT or BCG Δ pcaA either before or after exposure to an endocytic tracer and phagosomes were characterized in terms of presence or absence of lysosomal marker. C and F, immature loner phagosome devoid of lysosomal material; D, G, loner phagosome fusing (arrows) with lysosomes (L); E, H, social phagosome with lysosomal material. C–H, bar scale represents 0.5 μ m. I, fold increase in percentage of BCG Δ pcaA-containing loner phagosomes with lysosomal material with respect to BCG WT-containing loner phagosomes. J, percentage of loner phagosomes with lysosomal material (HRP) at 1 or 5 days post-infection. Both experiments (I, J) correspond to the means \pm S.E. (n = samples from 3 different donors).

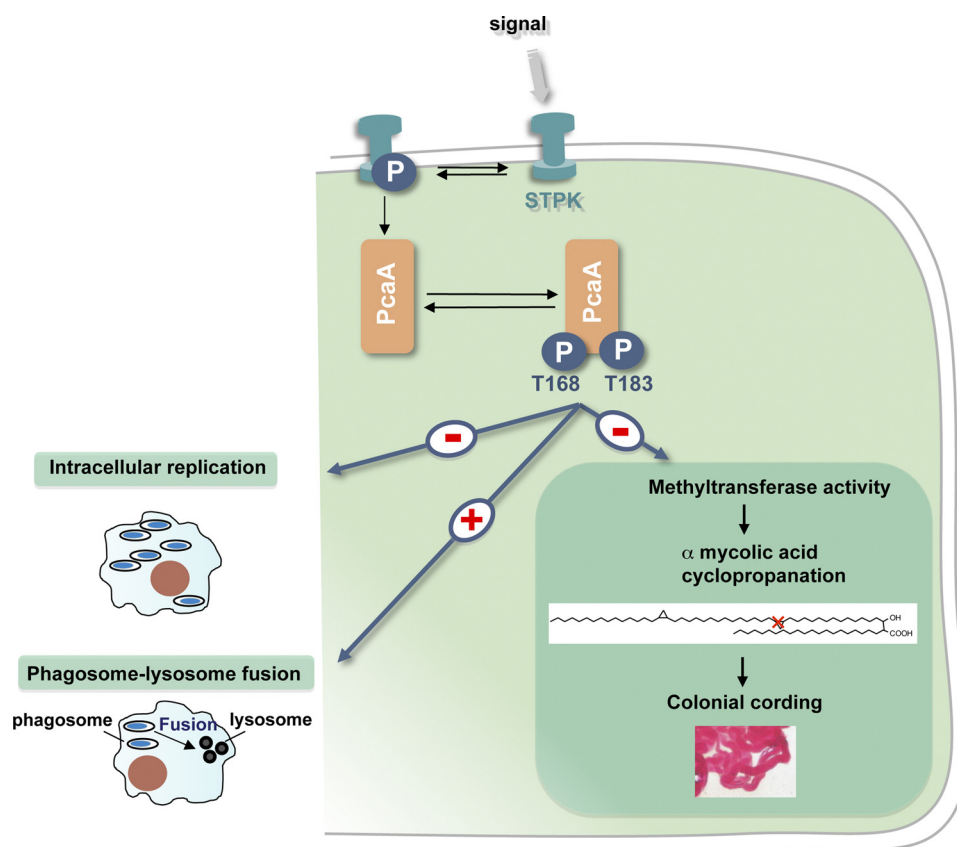


FIGURE 8. **Schematic representation of the consequences of STPK-dependent PcaA phosphorylation.** In response to external stimuli, STPKs are auto-phosphorylated. This induces PcaA phosphorylation on Thr-168 and Thr-183. PcaA phosphorylation decreases cyclopropane synthase activity, resulting in an altered mycolic acid profile characterized by the lack of di-cyclopropanated α -mycolic acids. This affects colonial cording, intramacrophage replication and abrogates the PMB.

phorylation is reduced/inhibited once the close apposition between the phagosome membrane and the mycobacterial surface is no longer maintained all around, cannot be determined with present technologies as it is not possible to separate the different types of phagosomes from one another.

For a successful infection, it must be assumed that pathogenic mycobacteria will not be destroyed in macrophages. In many situations, growth is arrested in phagolysosomes but mycobacteria survive in this cytolytic environment without significant loss of viability (2–4, 39). Our study is in full agreement with the above situations as shown by the survival curves and the morphological appearance of bacilli within phagolysosomes.

In summary, this study provides conceptual advances in our understanding of the mycolic acid metabolic adaptation and regulatory events exploited by pathogenic mycobacteria to adapt their mycolic acid cell wall content. Although very challenging, future studies should now help to identify extracellular cues sensed by the different kinases and leading to PcaA phosphorylation. This work also strengthens the biological importance of PcaA in the physiology and virulence of the bacilli and provides evidence of a Ser/Thr kinase-dependent mechanism for modulating the composition of mycolic acids, a key component of the mycobacterial cell wall, and for maintaining mycobacteria in a non-matured phagosome, a hallmark for mycobacterial survival within host cells. Our results suggest that displacement of the unphosphorylated/phosphorylated PcaA balance in favor of the phosphorylated isoform rapidly leads to PMB inhibition and loss

of intracellular survival. Thus, from an applied point of view and considering that PcaA has been proposed as a target for drug development against persistent bacilli (18, 47), the selective inhibition of PcaA activity through constitutive phosphorylation may strongly impair *Mtb* survival, opening new and original perspectives for future anti-tuberculosis drug development. It is indeed noteworthy that small molecules, such as bryostatin (48) can activate STPK, which may be of great therapeutic value in inhibiting *Mtb* intracellular growth.

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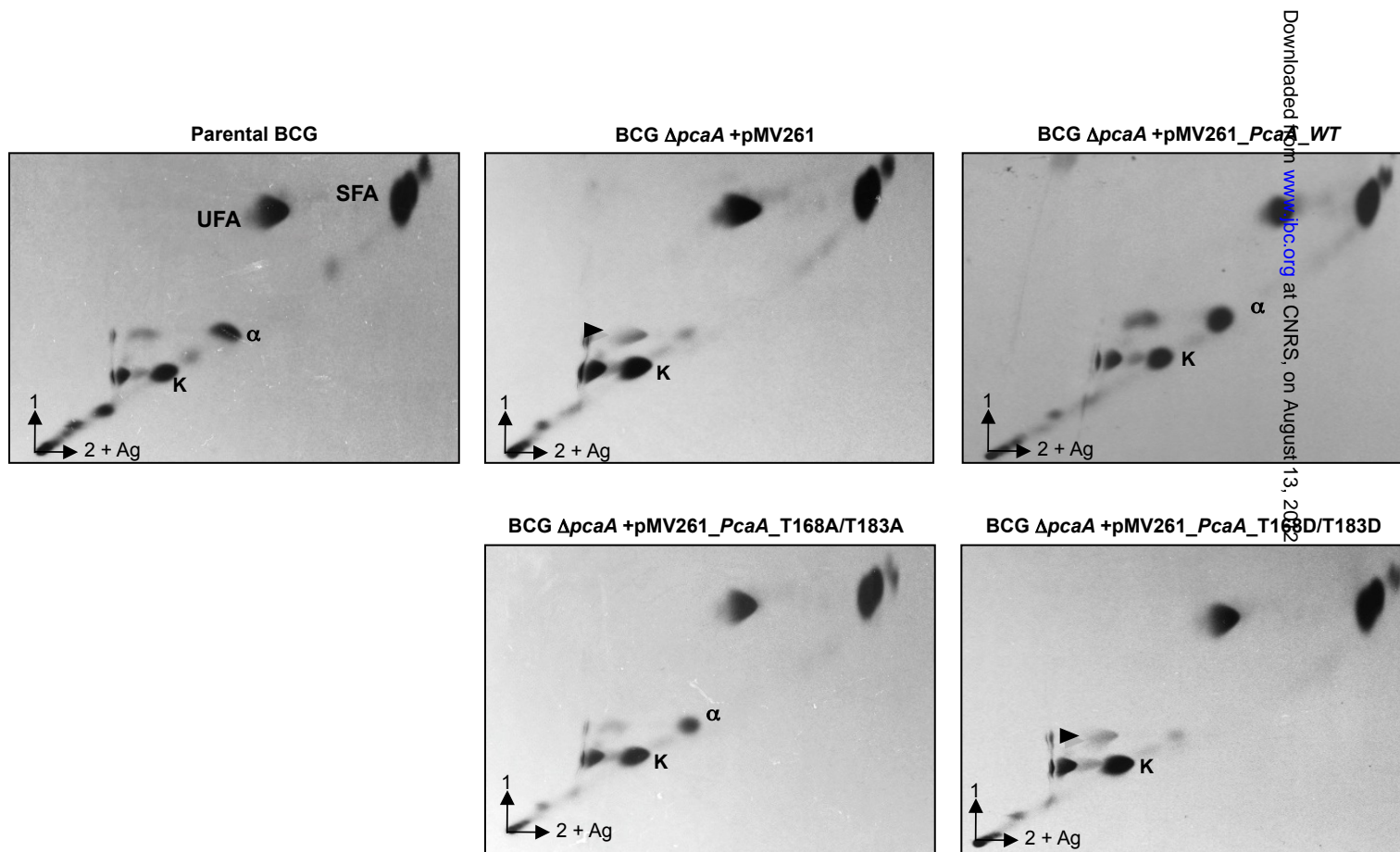


Fig. S1. Mycolic acid pattern in *M. bovis* BCG $\Delta pcaA$ complemented strains. Two-dimensional argention TLC of ^{14}C -radiolabeled mycolic acids from *M. bovis* BCG Pasteur and BCG mc²2801 (BCG Pasteur *pcaA*::Tn5370) transformed with various vectors and grown at 37°C. The arrowhead indicates accumulation of the unsaturated α -mycolic acid precursor concomitant to the loss of α -mycolic acid synthesis. This hybrid mycolate accumulating in the $\Delta pcaA$ mutant and in the mutant complemented with pMV261_ *PcaA*_T168D/T183D carries a *cis* double bond at the proximal position in place of a *cis* cyclopropane, as determined previously (Glickman *et al.* 2000). α , α -mycolates; K, keto-mycolates.

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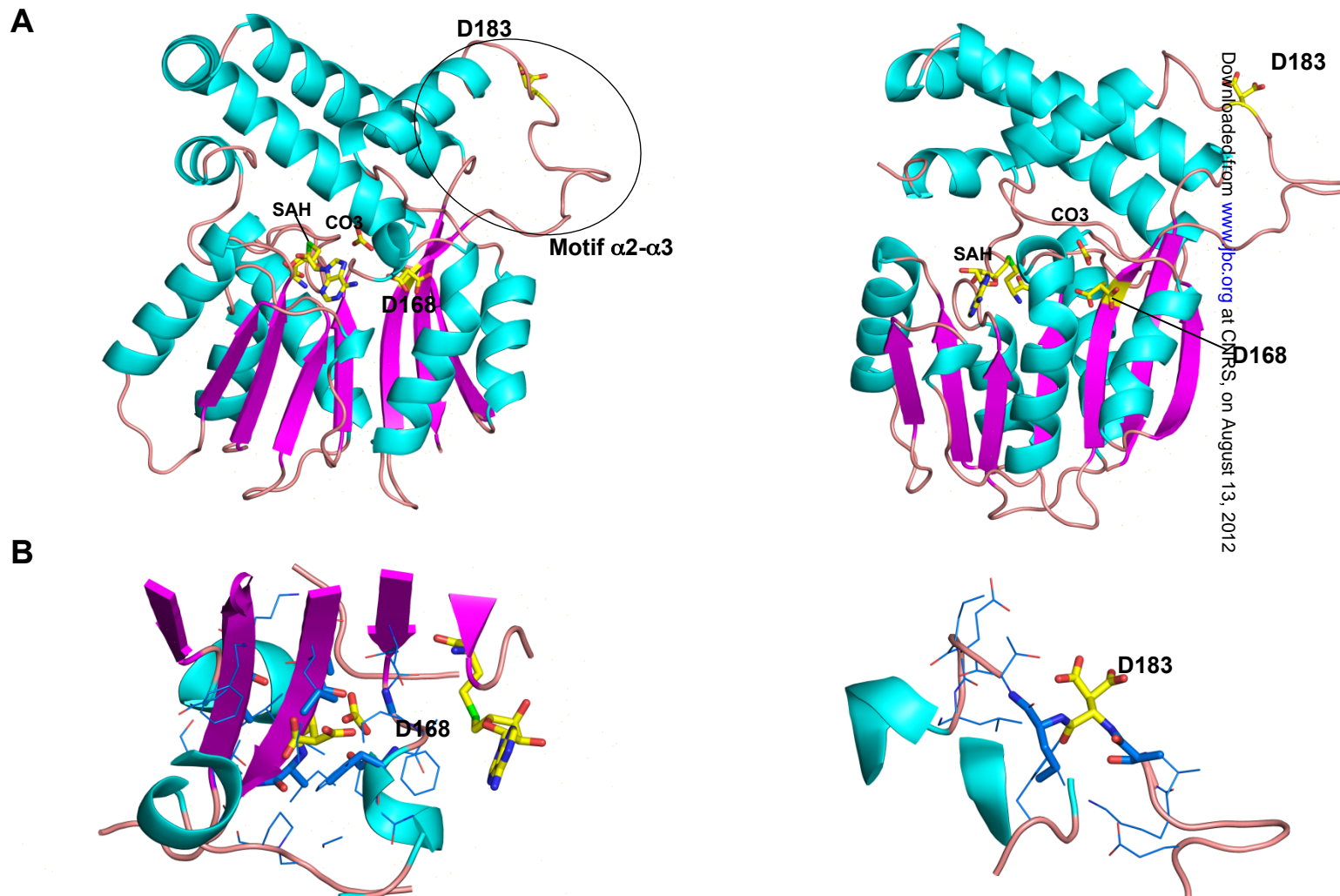


Fig. S2. Structure modeling of the PcaA phosphomimetic T168D/T183D mutant. (A) Overall views separated by 45°. (B) Zoom-in showing the chemical environment around D168 (left) and D183 (right): cartoon within a sphere of radius 13 Å, lines within a sphere of 8 Å, sticks within a sphere of 4 Å around these positions. The two most favorable side chain conformers of D168 and D183 are shown. SAH, the cofactor product, and the carbonate ion that was found in the active site are also displayed. α -Helices are in cyan, β -strands in magenta, turns and coils in salmon. Carbon atoms, yellow or marine; nitrogen atoms, blue; oxygen atoms, red; sulfur atoms, green. The Swiss-model program (www.swissmodel.expasy.org) (Arnold et al, 2006; Guex et al, 1997; Schwede et al, 2003) was used to deduce hypothetical structures from the sequences of PcaA mutants. The 1L1E.pdb structure was used as template and homology models were produced using the Automated mode. Structure visualization and analyses were carried out using the PyMOL program (www.pymol.org).

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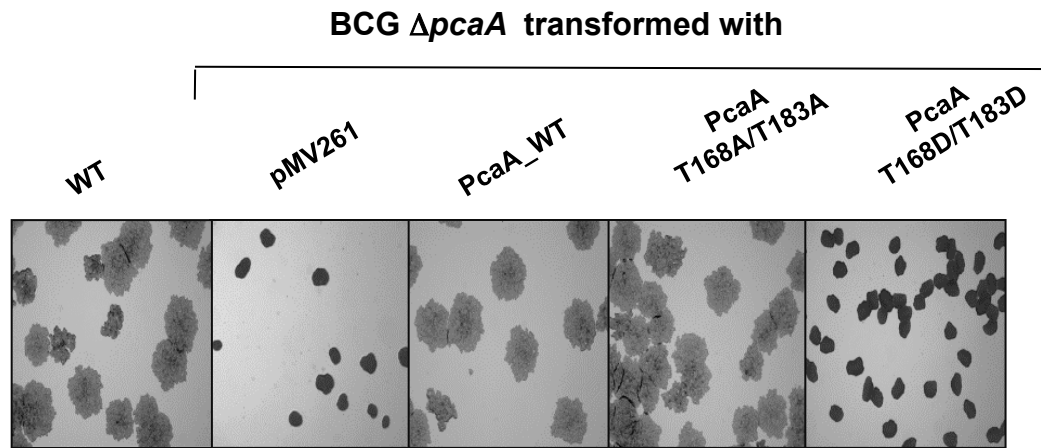


Fig. S3. Alteration of mycobacterial growth. *M. bovis* BCG $\Delta pcaA$ transformed with the various plasmids were plated on Middlebrook 7H10 supplemented with OADC and incubated at 37°C for 2-3 weeks.

Table S1. Bacterial Strains and Plasmids used in this study.

Strains or Plasmids	Genotype or Description	Source or Reference
<i>E. coli</i> DH5 α	<i>F'endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupGΦ80dlacZΔM15 Δ(lacZYA-argF) U169, hsdR17(r_K⁻ m_K⁺), λ- ; used for general cloning</i>	Invitrogen
<i>E. coli</i> C41 (DE3)	Strain F derived from BL21(DE3), used for expression of toxic proteins	Lucigen
<i>E. coli</i> BL21(DE3) Star	F2 <i>ompT hsdSB</i> (rB2 mB2) <i>gal dcm</i> (DE3); used to express recombinant proteins in <i>E. coli</i>	Invitrogen
<i>M. bovis</i> BCG 1173P2	Vaccine strain	WHO, Stockholm
mc ² 2801	BCG Pasteur <i>pcaA::Tn5370</i>	(1)
pETPhos	pET15b (Novagen) derivative including the replacement of the thrombin site coding sequence with a tobacco etch virus (TEV) protease site and Ser to Gly mutagenesis in the Nterm His-tag	(2)
pCDFDuet-1	pET vector derivative designed for the co-expression of two proteins under <i>T7lac</i> promoter induction	Novagen
pCDFDuet_ <i>pknF</i>	pCDFDuet-1 vector allowing expression of the PknF kinase domain untagged	(3)
pETPhos_ <i>MmaA2_WT</i>	pETPhos derivative used to express His-tagged fusion of WT <i>MmaA2</i> in <i>E. coli</i>	This work
pETPhos_ <i>PcaA_WT</i>	pETPhos derivative used to express His-tagged fusion of WT <i>PcaA</i> in <i>E. coli</i>	This work
pETPhos_ <i>PcaA_T168A</i>	pETPhos derivative used to express His-tagged fusion of <i>PcaA_T168A</i> in <i>E. coli</i>	This work
pETPhos_ <i>PcaA_T183A</i>	pETPhos derivative used to express His-tagged fusion of <i>PcaA_T183A</i> in <i>E. coli</i>	This work
pETPhos_ <i>PcaA_T168A/T168A</i>	pETPhos derivative used to express His-tagged fusion of <i>PcaA_T168A/T168A</i> in <i>E. coli</i>	This work
pVV16	<i>E. coli</i> /mycobacterial shuttle vector, allows expression of C-term His-tagged proteins, derived from pMV261 (Stover et al. 1991)	(4)
pVV16_ <i>PcaA_WT</i>	pVV16 derivative used to express His-tagged fusion of WT <i>PcaA</i> in mycobacteria	This work
pVV16_ <i>PcaA_T168A/T183A</i>	pVV16 derivative used to express His-tagged fusion of <i>PcaA_T168A/T183A</i> in mycobacteria	This work
pMV261	Multi-copy plasmid for <i>E. coli</i> and mycobacteria. Cloned genes are under the control of the constitutive <i>hsp60</i> promoter	(5)
pMV261_ <i>PcaA_WT</i>	pMV261 derivative used to express HA-tagged fusion of WT <i>PcaA</i> in mycobacteria	This work
pMV261_ <i>PcaA_T168A/T168A</i>	pMV261 derivative used to express HA-tagged fusion of WT <i>PcaA_T168A/T168A</i> in mycobacteria	This work
pMV261_ <i>PcaA_T168D/T168D</i>	pMV261 derivative used to express HA-tagged fusion of WT <i>PcaA_T168D/T168D</i> in mycobacteria	This work

Table S2. Primers used in this study.

Primers	5' to 3' Sequence ^{ab}
MmaA2 WT dir	CTACCTAC <u>CATATGGT</u> CAACGACCTAACGCCGCACTTC (NdeI)
MmaA2 WT rev	CATGGAT <u>GCTAGC</u> TACTTCGCCAGCGTGA <u>ACTGGT</u> (NheI)
PcaA WT dir	CTACCTAC <u>CATATGT</u> CCGTGCAGCTCACGCCGCAT(NdeII)
PcaA WT rev	CAGCGAGGGATCCTTACTTTTCCAGTGTGA <u>ACTGGT</u> CG (BamHI)
PcaA T168A dir	AAGATGTTGCTGCACGCCATCGTGCGCCCCACC
PcaA T168A rev	GGTGGGGCGCACGATGGCGTGCAGCAACATCTT
PcaA T183A dir	GGCAGGGAAAAGGGCCTGGCGTTGACCCACGA <u>ACTGGT</u>
PcaA T183A rev	AACCAGTTCGTGGGTCAACGCCAGGCCCTTTTCCCTGCC
pMV261_PcaA_WT dir	GTCCAGCGGTACCCGACAAGATCGGTTACGACG (KpnI)
pMV261_PcaA_WT rev	CGAGGAATTCTTAAGCGTAATCTGGAACATCGTATGGGTACTTTCCAGTGTGA <u>ACTGGT</u> (EcoRI)
pMV261_PcaA_T168D dir	CAAGATGTTGCTGCACGACATCGTGCGCCCCACC
pMV261_PcaA_T168D rev	GGTGGGGCGCACGATGTCGTGCAGCAACATCTTG
pMV261_PcaA_T183D dir	CAGGGAAAAGGGCCTGGACTTGACCCACGA <u>ACTGG</u>
pMV261_PcaA_T183D rev	CCAGTTCGTGGGTCAAGTCCAGGCCCTTTTCCCTG
pVV16_PcaA_WT rev	CAGCGCAAGCTTCTTTTCCAGTGTGA <u>ACTGGT</u> CG (HindIII)

^a Restriction sites are underlined and specified into brackets.

^b Mutagenized bases are shown in bold.

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