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The *Mycobacterium tuberculosis* β-Ketoacyl-Acyl Carrier Protein Synthase III Activity Is Inhibited by Phosphorylation on a Single Threonine Residue*[S]

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Mycolic acids are hallmark features of the *Mycobacterium tuberculosis* cell wall. They are synthesized by the condensation of two fatty acids, a C_{56-64}-meromycolyl chain and a C_{24-26}-fatty acyl chain. Meromycolates are produced via the combination of type I and type II fatty acid synthases (FAS-I and FAS-II). The β-ketoacyl-acyl carrier protein (ACP) synthase III (mtFabH) links FAS-I and FAS-II, catalyzing the condensation of FAS-I-derived acyl-CoAs with malonyl-ACP. Because mtFabH represents a potential regulatory key point of the mycolic acid pathway, we investigated the hypothesis that phosphorylation of mtFabH controls its activity. Phosphorylation of proteins by Ser/Thr protein kinases (STPKs) has recently emerged as a major physiological mechanism of regulation in prokaryotes. We demonstrate here that mtFabH was efficiently phosphorylated *in vitro* by several mycobacterial STPKs, particularly by PknF and PknA, as well as *in vivo* in mycobacteria. Analysis of the phosphoamino acid content indicated that mtFabH was phosphorylated exclusively on threonine residues. Mass spectrometry analyses using liquid chromatography-electrospray ionization/tandem mass spectrometry identified Thr^{45} as the unique phosphoacceptor. This was further supported by complete loss of PknF- or PknA-dependent phosphorylation of a mtFabH mutant. Mapping Thr^{45} on the crystal structure of mtFabH illustrates that this residue is located at the entrance of the substrate channel, suggesting that the phosphate group may alter accessibility of the substrate and thus affect mtFabH enzymatic activity. A T45D mutant of mtFabH, designed to mimic constitutive phosphorylation, exhibited markedly decreased transacylation, malonyl-AcpM decarboxylation, and condensing activities compared with the wild-type protein or the T45A mutant. Together, these findings not only represent the first demonstration of phosphorylation of a β-ketoacyl-ACP synthase III enzyme but also indicate that phosphorylation of mtFabH inhibits its enzymatic activity, which may have important consequences in regulating mycolic acid biosynthesis.

Within the infected host, *Mycobacterium tuberculosis* encounters numerous environmental conditions and induces or represses a number of genes for a quick adjustment to new conditions. The infection process of *M. tuberculosis* involves cross-talk of signals between the host and the bacterium, resulting in reprogramming of the host signaling network. Protein phosphorylation/dephosphorylation represent a central mechanism for distribution of signals to various parts of the cell, regulating growth, differentiation, mobility, and survival (1). In mycobacteria, a common signal transduction pathway is transmitted through membrane-embedded sensor kinases, enabling the pathogen to modify itself for survival in this hostile environment.

Protein kinases can be classified into two families based on their similarities and enzymatic specification: (i) the histidine kinase superfamily, which relies on autophosphorylation of a conserved histidine residue and commonly occurs as part of two-component systems and (ii) the superfamily of serine, threonine, and tyrosine kinases that phosphorylate on serine, threonine, and tyrosine residues, respectively (1–3). Although the two-component systems represent the classical prokaryotic mechanism for detection and response to environmental changes, the serine/threonine/tyrosine protein kinases and phosphatases are also widespread in prokaryotes. The *M. tuberculosis* genome contains 11 Ser/Thr protein kinases (STPKs)[6]

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[6] The abbreviations used are: STPK, Ser/Thr protein kinase; ACP, acyl carrier protein; FAS-I, eukaryotic type I fatty acid synthase; FAS-II, prokaryotic type II fatty acid synthase; PDIM, phthiocerol dimycocerosate; GST, glutathione *S*-transferase; KAS, β-ketoacyl-ACP synthase; MS, mass spectrometry; mtFabH, *M. tuberculosis* β-ketoacyl-acyl carrier protein synthase III.

(4, 5), and most are being investigated for their physiological roles and potential application for future drug development to combat tuberculosis (6). This significant number of STPKs suggests that phosphorylation may influence a wide range of biological functions, such as adaptation to various environmental conditions, stress, cell wall synthesis, cell division, and pathogenicity (7–14). The cell wall of *M. tuberculosis* plays a critical role in the defense of this pathogen in the host, since environmental stimuli induce changes in cell wall composition and thus help *M. tuberculosis* to adapt during infection (15, 16). Several recent studies support the view that regulation of cell wall synthesis involves Ser/Thr kinases. For instance, PknH phosphorylates the FHA-containing protein EmbR, a putative transcriptional regulator of the EmbCAB arabinosyltransferases (17). Activation of EmbR upon phosphorylation by PknH induces transcription of the *embCAB* operon, leading to a higher lipoarabinomannan/lipomannan ratio (18), and regulates the synthesis of arabinan, an important component of arabinogalactan, essential for the structural integrity of the cell wall. Also, lipomannan and lipoarabinomannan are major cell wall-associated lipoglycans and important modulators of the immune system (19). Furthermore, a recent study has shown that PknH was able to phosphorylate other substrates, such as DacB1 (20). In *Bacillus subtilis*, DacB1 is a sporulation-specific protein involved in cell envelope biosynthesis. Based on homology with the *B. subtilis* orthologue, it has been postulated that PknH-dependent phosphorylation of *M. tuberculosis* DacB1 regulates the synthesis of peptidoglycan, a key component of the mycobacterial cell envelope (20). Finally, MmpL7, a member of the RND (resistance, nodulation, and cell division) family of transporters that is thought to be involved in the transport of phthiocerol dimycocerosate and known to be essential for virulence (21, 22), has been shown to undergo phosphorylation. The last finding suggests that kinases could also play an important role in regulating the transport of cell wall components (23).

In view of the large repertoire of polyketides and complex lipids present in *M. tuberculosis*, STPKs could play an important role in regulating the metabolism of these molecules. There is now evidence to suggest that phosphorylation may also participate in the regulation of mycolic acids. The β-ketoacyl-ACP synthases KasA and KasB of the mycobacterial FAS-II system involved in fatty acid elongation represent STPKs substrates, and phosphorylation of KasA and KasB differentially modulates their condensing activity *in vitro* (24). The present study was undertaken to address the question of whether mycolic acid synthesis in general might be influenced by STPK-dependent regulatory mechanisms. Here, we have identified mtFabH as a new substrate of the *M. tuberculosis* SPTKs and defined its phosphorylation state. This allowed us to address the role/contribution of phosphorylation in the enzyme activity.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—Strains used for cloning and expression of recombinant proteins were *Escherichia coli* TOP10 (Invitrogen) and *E. coli* BL21(DE3)pLysS (Novagen). Strains were grown at 37 °C in LB medium supplemented with 100 μg/ml ampicillin or 25 μg/ml kanamycin when required. *M. bovis* BCG Pasteur 1173P2 strain was plated on Middlebrook 7H10 agar supplemented with OADC enrichment (Difco) and 25 μg/ml kanamycin when required or grown in Sauton's medium containing 0.05% tyloxapol (Sigma).

Cloning, Expression, and Purification of mtFabH and Mutant Proteins—The *mtfabH* gene was amplified by PCR using *M. tuberculosis* H37Rv chromosomal DNA as a template and the primers 5′-TAA TAG CTC ATA TGA CGG AGA TCG CCA CGA CCA GC-3′ and 5′-TAA TAG CTG CTA GCT CAA CCC TTC GGC ATT CGC AC-3′ containing an NdeI or NheI restriction site, respectively (underlined). This 1008-bp amplified product was then digested by NdeI and NheI and ligated into the pETTev plasmid, a variant of pET15b (Novagen) that includes the replacement of the thrombin site coding sequence with a tobacco etch virus protease site (25), thus generating pETTev-*mtfabH*. Site-directed mutagenesis was directly performed on pETTev-*mtfabH* using inverse PCR amplification with the following self-complementary primers: 5′-CGT CCG ACG AGT GGA TCT AC**G** CCC GAA CCG GCA TCA AGA C-3′ and 5′-GTC TTG ATG CCG GTT CGG G**C**G TAG ATC CAC TCG TCG GAC G-3′ for mtFabH_T45A and 5′-CGT CCG ACG AGT GGA TCT AC**G** **A**CC GAA CCG GCA TCA AGA C-3′ and 5′-GTC TTG ATG CCG GTT CGG **TC**G TAG ATC CAC TCG TCG GAC G-3′ for mtFabH_T45D (the corresponding substitutions are shown in boldface type). All constructs were verified by DNA sequencing. Recombinant strains harboring the mtFabH-expressing constructs were used to inoculate 200 ml of LB medium supplemented with ampicillin, and resulting cultures were incubated at 37 °C with shaking until A_{600} reached 0.5. isopropyl 1-thio-β-D-galactopyranoside (1 mM final) was added, and growth was continued for 3 h at 37 °C. Purifications of mtFabH_WT, mtFabH_T45A, and mtFabH_T45D were performed as described earlier (24).

Overexpression and Purification of mtFabH in M. bovis BCG—The *M. tuberculosis* H37Rv *mtfabH* gene was amplified using the following primers: pVV16-*mtfabH*-up (5′-GAT AGG ACG CAT ATG ACG GAG ATC-3′) and pVV16-*mtfabH*-lo (5′-AAG CTT ACC CTT CGG CAT TCG CAC CAC-3′) (containing an NdeI and HindIII site, respectively). The PCR product was cut with NdeI/HindIII, enabling direct cloning into the pVV16 expression vector cut with the same enzymes (26). This plasmid harbors the *hsp60* promoter as well as a His tag for expression of C-terminal His-tagged fusion proteins. The resulting vector, pVV16-*mtfabH*, was used to transform *M. bovis* BCG. Purification of soluble mtFabH-His_6 was performed on Ni^{2+}-nitrilotriacetic acid-agarose beads, as described previously (24).

In Vitro Kinase Assay—*In vitro* phosphorylation was performed as described earlier (17) with 2 μg of mtFabH in 20 μl of buffer P (25 mM Tris-HCl, pH 7.0, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1 mM EDTA) with 200 μCi/ml [γ-^{33}P]ATP corresponding to 65 nM (3000 Ci/mmol; PerkinElmer Life Sciences) and 0.6–4.2 μg of kinase in order to obtain for each specific kinase its optimal autophosphorylation activity for 30 min at 37 °C. Cloning, expression, and purification of the eight recombinant GST-tagged STPKs from *M. tuberculosis* were described previously (24).

Analysis of the Phosphoamino Acid Content of mtFabH—mtFabH samples phosphorylated *in vitro* in the presence of GST-tagged PknF (or PknA) and [γ-^{33}P]ATP were separated by one-dimensional gel electrophoresis and electroblotted onto an Immobilon polyvinylidene difluoride membrane. Analysis of the phosphoamino acid content was done as reported previously (17).

Mass Spectrometry Analysis—Purified mtFabH_WT and mtFabH_T45A proteins were subjected to *in vitro* phosphorylation by GST-tagged PknF or PknA as described above, except that [γ-^{33}P]ATP was replaced with 5 mM cold ATP. Subsequent mass spectrometry analyses were performed as previously described (27).

Two-dimensional Gel Electrophoresis and Western Blot Analysis—M. bovis BCG crude lysates were prepared as described earlier (24). Approximately 150 μg of total soluble proteins were loaded onto a 7-cm immobiline strip (Bio-Rad, pH 3.9–5.1) and electrophoresed in a Protean IEF Cell (Bio-Rad) in the first dimension and on a 10% SDS-PAGE in the second dimension. Proteins were then blotted on a polyvinylidene difluoride membrane and probed with a rabbit anti-mtFabH antibody raised against recombinant mtFabH (1:250 dilution). The membrane was then incubated with horseradish peroxidase-conjugated anti-rabbit antibodies (1:5000 dilution), and detection of the different mtFabH isoforms was carried out using the Western Lightening Reagent (PerkinElmer Life Sciences) according to the manufacturer's instructions.

mtFabH Condensation Assay—The condensing activity of mtFabH was assayed by mixing 50 μM holo-AcpM (mycobacterial acyl carrier protein), 1 mM β-mercaptoethanol, 0.1 M sodium phosphate buffer, pH 7.0, 50 μM malonyl-CoA, 45 nCi of [2-^{14}C]malonyl-CoA (specific activity, 55 Ci/mol), 12.5 μM palmitoyl-CoA, and 0.3 μg of mtFabD in a final volume of 40 μl. The mtFabD protein was added to generate the malonyl-AcpM substrate for the reaction *in situ*. A mixture of holo-AcpM containing 1 mM β-mercaptoethanol was incubated at 37 °C for 30 min to ensure complete reduction of AcpM, prior to the addition of the remaining components (except mtFabH). The reaction was initiated by the addition of 0.5 μg of mtFabH, held at 37 °C for 40 min, and quenched by adding 5 mg/ml $NaBH_4$ in 100 mM K_2HPO_4, 100 mM KCl, 30% tetrahydrofuran. This resulted in the liberation of β-ketoacyl groups from their respective thioesters as acyl-1,3-diols, which were extracted with water-saturated toluene. The radiolabeled products were then quantified by liquid scintillation counting.

mtFabH Transacylation Assay—Acyl transfer was assessed using [9,10-^{3}H]myristoyl-CoA (specific activity 57.0 Ci/mmol) to follow the transacylation of the acyl chain from acyl-CoA to the active site Cys^{122} of mtFabH. The reaction mixture consisted of 50 mM Tris/HCl, pH 7.5 (Buffer A), 250 pmol of [^{3}H]myristoyl-CoA (14.6 nCi), and 4 μg of WT or mutant mtFabH (110 pmol) in a final volume of 60 μl. The reaction was held at room temperature for 15 min, and 200 μl of Ni^{2+}-charged chelating Sepharose fast flow chromatography medium (50% slurry in buffer A) was added. After a further 5 min, the slurry was diluted by adding 300 μl of buffer A, and unbound C_{14}-CoA was then removed by filtration (cellulose nitrate, 0.45 μm). The beads were washed with 20 ml of buffer A before the filters were removed to vials for liquid scintillation counting.

Malonyl-AcpM Decarboxylation Assay—The methods used by Brown *et al.* (28) and Shorrosh *et al.* (29) have been combined and modified to investigate the decarboxylation of malonyl-CoA by mtFabH_WT and mutants. Malonyl-AcpM was synthesized from malonyl-CoA and holo-AcpM *in situ* using mtFabD. The initial reaction mixture of 25 mM Tris/HCl, pH 7.5, 12 μM AcpM, and 1 mM dithiothreitol in a volume of 28 μl was held at 4 °C for 30 min. 160 nCi of [2-^{14}C]malonyl-CoA and 0.3 μg of mtFabD were added and incubated at 37 °C for 1 h prior to the addition of 5 μg of either mtFabH_WT or mutants. The reaction was quenched after 1.5 h at 37 °C by the addition of 900 μl of ice-cold 10% (w/v) trichloroacetic acid at 4 °C and 50 μl of 2 mg/ml bovine serum albumin and incubated on ice for 20 min. The solution was centrifuged at 27,000 $\times$ *g* for 15 min, and the protein pellets were washed with 900 μl of ice-cold 10% (w/v) trichloroacetic acid and centrifuged. The pellet was dried to remove excess trichloroacetic acid and then resuspended in 40 μl of 50 mM Tris/HCl, pH 7.4. 10 μl of 2 M hydroxylamine (pH 7.0) was added to form hydroxamate derivatives of malonyl-CoA and acetyl-CoA and incubated at 37 °C for 1 h. 5 μl of the resulting mixture was subjected to scintillation counting, and the remaining 45 μl was spotted on 10 $\times$ 20-cm Cellulose 300, F-254 glass-backed TLC plates (Fisher), where [^{14}C]acetyl-hydroxamate and [^{14}C]malonyl-hydroxamate were separated by butanol/acetic acid/water (8:2:3, v/v/v). Autoradiograms were obtained after exposing the plates for 7 days to Kodak X-Omat AR film.

RESULTS

mtFabH Is a Substrate of Mycobacterial STPKs—The coupling of the acyl and malonyl groups to form β-ketoacyl derivatives is catalyzed by the β-ketoacyl-ACP synthase (KAS) components of the FAS-II system (30, 31). Three distinct KASs, mtFabH (KAS-III), KasA, and KasB, operate in the *M. tuberculosis* FAS-II pathway. The mtFabH enzyme has been reported to act as the pivotal link between FAS-I and FAS-II by initiating the biosynthesis of very long chain fatty acids used in mycolate biosynthesis (32). It catalyzes the condensation of an acyl-CoA substrate (a FAS-I end product) with malonyl-AcpM to generate a β-ketoacyl-AcpM product (32), which following reduction generates an acyl-AcpM with two more carbons. KasA and KasB are β-ketoacyl-AcpM synthases that further elongate the growing acyl chain in the mycobacterial FAS-II system (33–36) and have been shown to undergo post-translational modification by phosphorylation (24). Herein, we investigated whether mtFabH would also represent a substrate for mycobacterial kinases. STPKs of *M. tuberculosis* (PknA, PknB, PknD, PknE, PknF, PknH, PknK, or PknL) were expressed as GST fusions and purified from *E. coli* as described previously (24). Recombinant mtFabH was expressed and purified from *E. coli* BL21(DE3)pLysS harboring the pETTev-*mtfabH*. The protein contained a N-terminal His tag, which was subsequently removed following cleavage with the tobacco etch virus protease. Interestingly, when the different STPKs were incubated in the presence of mtFabH and [γ-^{33}P]ATP, phosphorylation of mtFabH was observed, although levels varied for different

kinases. PknA and PknF were the most efficient kinases to phosphorylate mtFabH (Fig. 1*A*). PknD, PknE, and PknH, which display strong autophosphorylation activity *in vitro*, phosphorylated mtFabH to a lesser extent than PknA or PknF. PknB, PknK, and PknL did not phosphorylate mtFabH, which may be due, at least for PknK and PknL, to their very low autokinase activity. STPKs usually migrate as diffuse bands, reflecting the different levels of phosphorylation for each isoform, and this aberrant profile of migration of *M. tuberculosis* kinases has already been reported in earlier studies (17, 37–39). These results are consistent with the fact that KasA and KasB were also found to be efficiently phosphorylated by PknA and PknF (24). In contrast, although PknB efficiently phosphorylates KasA and KasB, it failed to phosphorylate mtFabH *in vitro* (Fig. 1*A*). Together, these data suggest different levels of substrate specificity of the various STPKs.

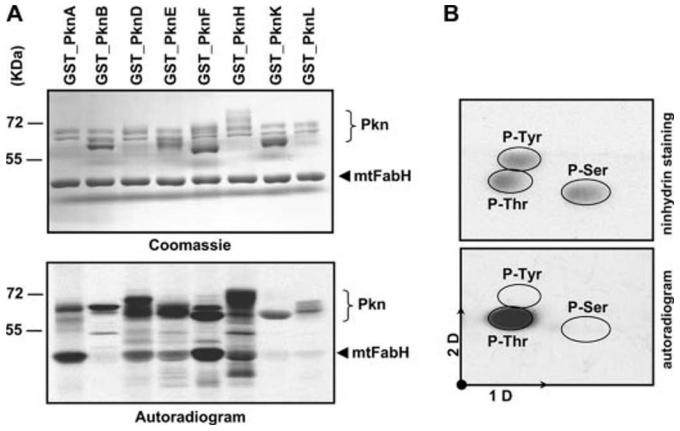


FIGURE 1. ***In vitro* phosphorylation of mtFabH.** *A*, *in vitro* phosphorylation of mtFabH by mycobacterial STPKs. Eight recombinant STPKs (PknA–PknL) encoded by the *M. tuberculosis* genome were expressed and purified as GST fusions and incubated with purified His-tagged mtFabH and [γ-^{33}P]ATP. The quantity between the STPKs was varied from 0.6 to 4.2 μg in order to obtain for each specific kinase its optimal autophosphorylation activity. Samples were separated by SDS-PAGE (*top*) and visualized by autoradiography (*bottom*). The *upper bands* illustrate the autokinase activity of each STPK, whereas *lower bands* reflect phosphorylation of mtFabH. *B*, phosphoamino acid content of mtFabH. mtFabH was phosphorylated *in vitro* in the presence of PknF and [γ-^{33}P]ATP, analyzed by SDS-PAGE, electroblotted onto an Immobilon polyvinylidene difluoride membrane, excised, and hydrolyzed in acid. The phosphoamino acids thus liberated were separated by electrophoresis in the first dimension (*1D*) and ascending chromatography in the second dimension (*2D*). After migration, radioactive molecules were detected by autoradiography (*bottom*). Authentic phosphoserine (*P-Ser*), phosphothreonine (*P-Thr*), and phosphotyrosine (*P-Tyr*) were run in parallel as internal standard controls and visualized by ninhydrin staining (*top*).

PknF Phosphorylates mtFabH in Vitro on Threonine Residues—To examine the nature of the mtFabH-phosphorylated residues, we analyzed the phosphoamino acid content of PknF-phosphorylated mtFabH. The mtFabH protein (3 μg) was labeled with [γ-^{33}P]ATP *in vitro*, separated by SDS-PAGE, excised, and subjected to acid hydrolysis. mtFabH was exclusively phosphorylated on threonines (Fig. 1*B*). Similar results were obtained when mtFabH was phosphorylated with PknA (data not shown). Understanding the potential regulatory role that phosphorylation of a given kinase substrate might play necessitates the identification of the specific sites undergoing phosphorylation. Although experimentally challenging, liquid chromatography/MS/MS mass spectrometry allowed us to decipher the different phosphorylation sites in a sequence-specific fashion.

mtFabH Is Phosphorylated on a Unique Threonine Residue—To identify which of the 22 threonines of mtFabH correspond to the phosphorylated site(s), recombinant mtFabH was incubated with cold ATP in the presence of PknF and subjected to mass spectrometry analysis after tryptic digestion. ProteinPilot® data base searching software (version 2.0; Applied Biosystems), using the Paragon method with phosphorylation emphasis, was used to detect and identify the phosphorylated peptides. The sequence coverage of the protein was 98%, and phosphorylation occurred only on peptide (26–50) with an 80 Da mass increment from 2978.41 to 3058.39 Da (monoisotopic mass). The MS/MS spectrum of the corresponding triply charged ion at *m/z* 1020.47 unambiguously confirmed the presence of the phosphate group on the threonine residue Thr[45] (Fig. 2), consistent with phosphoamino analysis (Fig. 1*B*). The absence of mass increments lower or higher than 80 Da excludes the eventuality of any other post-translational modifications on mtFabH.

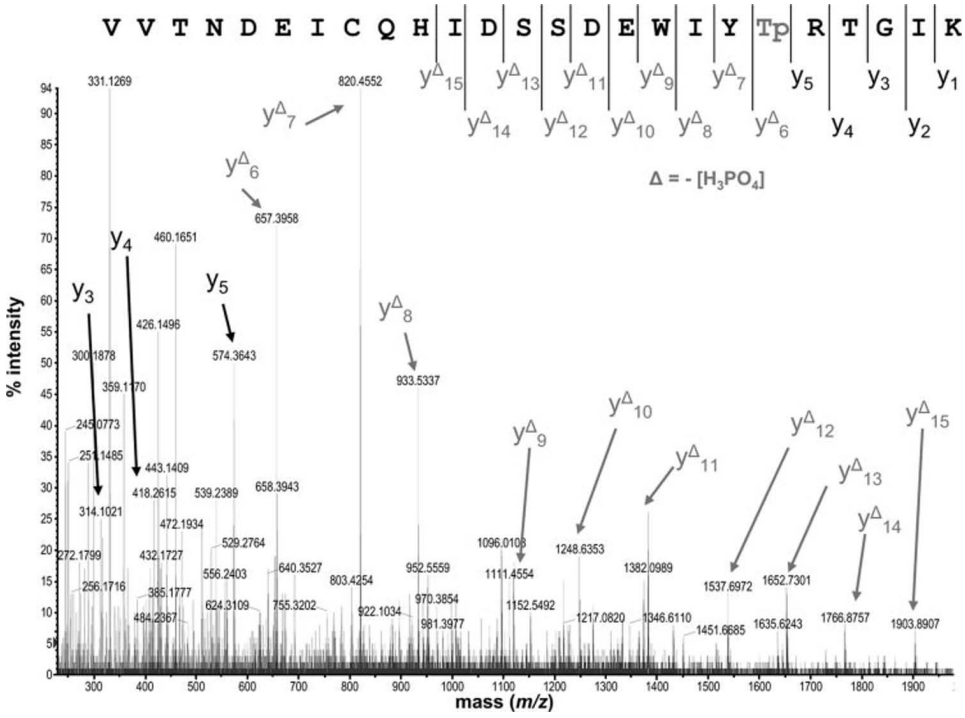


FIGURE 2. **MS/MS spectrum of the triply charged ion [M + 3H]$^{3+}$ at *m/z* 1020.47 of peptide 26–50 (monoisotopic mass 3058.39 Da).** The unambiguous location of the phosphate group on Thr[45] was shown by observation of the "y" C-terminal daughter ion series. Starting from the C-terminal residue, all y ions lose phosphoric acid (−98 Da) after the Thr[45] phosphorylated residue.

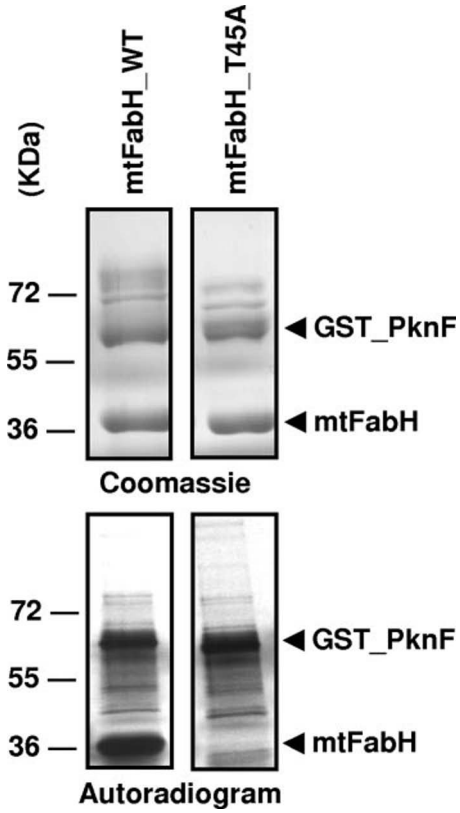


FIGURE 3. ***In vitro* phosphorylation of the mtFabH_T45A mutant.** Purified mtFabH_WT and mtFabH_T45A were incubated with PknF and [γ-^{33}P]ATP. Samples were separated by SDS-PAGE (*top*) and visualized by autoradiography (*bottom*).

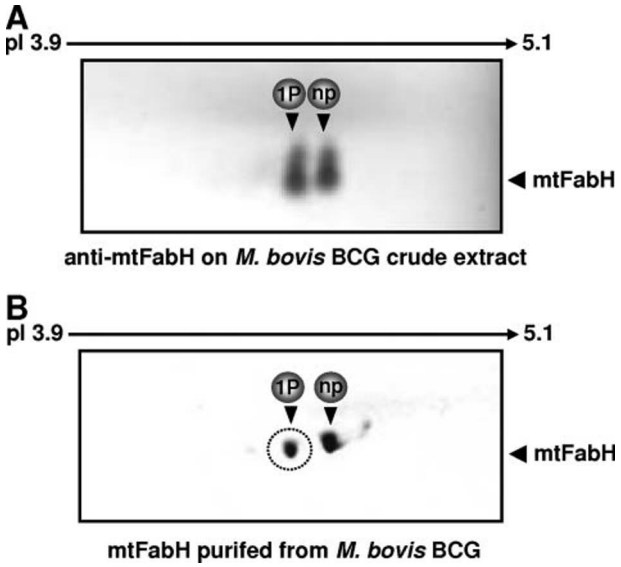


FIGURE 4. ***In vivo* phosphorylation of mtFabH in *Mycobacterium bovis* BCG.** *A*, detection of phosphorylated mtFabH in *M. bovis* BCG crude lysates. Approximately 150 μg of total soluble proteins from *M. bovis* BCG lysate were loaded on a 7-cm immobiline strip (pH 3.9–5.1; Bio-Rad) and electrophoresed on a Protean IEF Cell (Bio-Rad) for the first dimension and on a 10% SDS-PAGE for the second dimension. Proteins were then transferred to a polyvinylidene difluoride membrane and probed with rabbit antibodies raised against the *M. tuberculosis* mtFabH protein. Following incubation with Horseradish peroxidase-conjugated anti-rabbit serum as secondary antibody, detection was carried out using the Western Lightening Reagent (PerkinElmer Life Sciences). A selected portion of the membrane that strongly reacted with the antibodies, corresponding to mtFabH (34.8 kDa, pI 4.8), is represented. *B*, phosphorylation profile of mtFabH purified from recombinant *M. bovis* BCG carrying the pVV16-*mtfabH* construct. Cultures were recovered and lysed, and the soluble fraction was incubated with Ni^{2+}-nitrilotriacetic acid-agarose beads in order to purify His-tagged mtFabH, which was then subjected to two-dimensional gel electrophoresis and stained with Coomassie Blue. The *dashed circle* indicates the position of the spot that was subsequently excised from the polyacrylamide gel and analyzed by mass spectrometry. The *arrowheads* representing the different phosphorylated isoforms are indicated. *np*, nonphosphorylated; *1P*, monophosphorylated.

Definitive identification and localization of Thr^{45} as being the unique phosphorylation site in mtFabH was achieved by site-directed mutagenesis to introduce a mutation that prevents specific phosphorylation (Thr^{45} $\rightarrow$ Ala replacement). This mutant was expressed, purified as a His-tagged protein in *E. coli* BL21(DE3)pLysS harboring pETTev-*mtfabH(T45A)*, and used in an *in vitro* kinase assay. After cleavage of the His tag by the tobacco etch virus protease, the recombinant mtFabH_T45A was incubated along with [γ-^{33}P]ATP and PknF. The mixture was separated by SDS-PAGE and analyzed by autoradiography. As shown in Fig. 3 (*upper panel*), equal amounts of mtFabH_WT or mutant mtFabH_T45A were used. Phosphorylation of mtFabH_T45A was completely abrogated, compared with phosphorylation of mtFabH_WT, as evidenced by the absence of a specific radioactive band (Fig. 3, *lower panel*). These results unambiguously demonstrate that mtFabH_T45A has lost its ability to be phosphorylated by PknF. An additional round of mass spectrometry analysis was also performed directly on mtFabH_T45A pretreated with ATP and PknF, which failed to identify any additional phosphate group that could eventually have arisen as a compensatory mechanism to the loss of the Thr^{45} phosphorylation (data not shown).

In order to investigate whether Thr residue(s) different from Thr^{45} could be phosphorylated by other STPKs, mtFabH was incubated with [γ-^{33}P]ATP in the presence of PknA, PknD, PknE, or PknH, which are all able to phosphorylate mtFabH *in vitro* (Fig. 1*A*). In fact, the T45A substitution completely abrogated *in vitro* phosphorylation of mtFabH_T45A by PknA/D/E/H in comparison with mtFabH_WT (data not shown), indicating that Thr^{45} represents the unique phosphorylation site for all kinases. To our knowledge, this is the first demonstration that different mycobacterial kinases can interact and phosphorylate identical phosphorylation site in a given substrate.

mtFabH Is Phosphorylated in Vivo in M. bovis BCG on Thr^{45}—To assess the relevance of *in vitro* phosphorylation, we investigated the *in vivo* phosphorylation pattern of mtFabH in *M. bovis* BCG Pasteur. A proteomic approach was adopted in which total soluble *M. bovis* BCG proteins were resolved on a two-dimensional gel, subsequently transferred to a membrane, and probed with antibodies raised against *M. tuberculosis* FabH. Two spots were detected in a pH range close to the predicted pI of 4.8 of mtFabH (Fig. 4*A*). These spots presumably correspond to two mtFabH isoforms differing by a translational modification of the protein. Since a phosphate group renders the total charge of a protein more negative, a phosphorylated protein is expected to migrate toward a more acidic pH on a two-dimensional gel. Thus, the two isoforms presumably correspond to the nonphosphorylated (*np*) and the monophosphorylated (*1P*) isoforms of mtFabH (Fig. 4*A*).

To further support *in vivo* phosphorylation of mtFabH, we have designed a recombinant *M. bovis* BCG strain allowing the

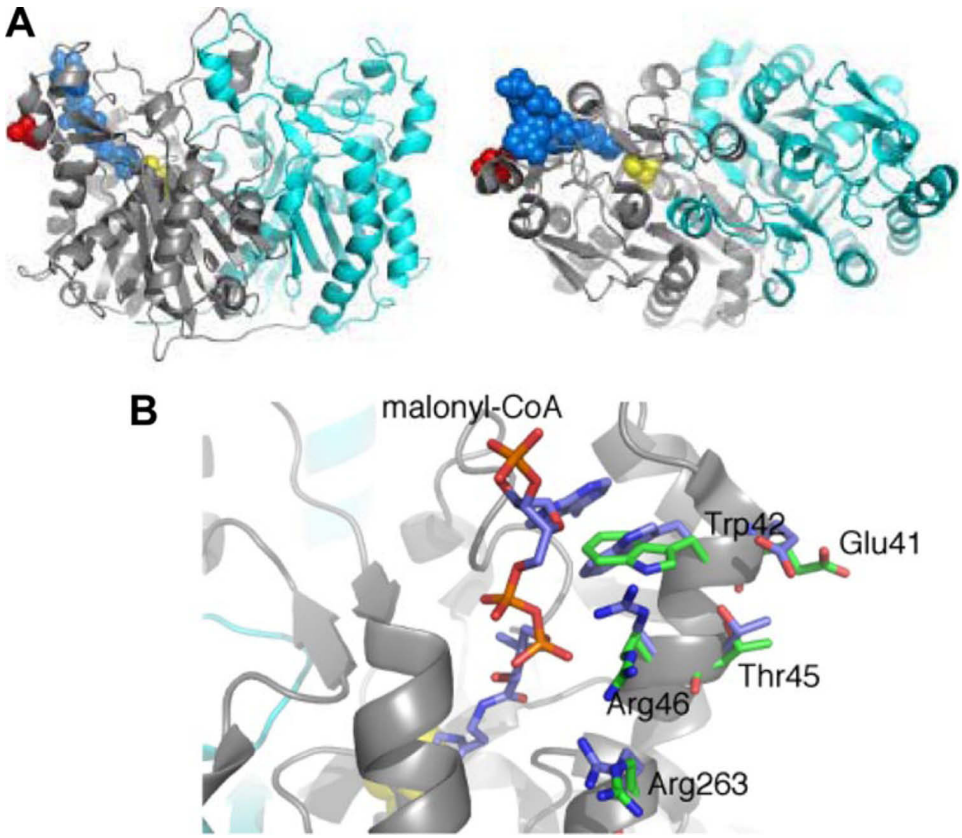


FIGURE 5. **Mapping of the Thr^{45} phosphorylation site on the mtFabH crystal structure.** *A*, two orthogonal views of the mtFabH dimer (Protein Data Bank entry 1M1M (28); *gray* and *cyan ribbon*) superimposed with malonyl-CoA-bound *E. coli* FabH (Protein Data Bank entry 1HNJ (43)) illustrating, for one protomer, the respective locations of the active site cysteine (*yellow spheres*), the substrate channel (malonyl-CoA in *blue spheres*), and Thr^{45} (*red spheres*). *B*, close-up view of the mtFabH substrate channel, demonstrating surface exposure of Thr^{45} and its proximity to mtFabH residues Arg^{46} and Trp^{42}. *Blue sticks* represent residues of *E. coli* FabH bound to malonyl-CoA (Protein Data Bank entry 1HNJ (EcFabH); *green sticks* show the corresponding side chains of mtFabH (Protein Data Bank entry 1M1M). Residue numbers refer to the amino acid sequence of mtFabH.

overexpression of a soluble His-tagged version of the protein. Cultures of *M. bovis* BCG harboring pVV16-*mtfabH* were collected, lysed, and fractionated in order to separate the soluble cytosolic compartment from the cell envelope. Soluble mtFabH was then purified to homogeneity by affinity chromatography on nickel-containing beads under native conditions and subjected to two-dimensional gel electrophoresis. As shown in Fig. 4*B*, two spots, presumably corresponding to the nonphosphorylated form, followed in the acidic direction by a monophosphorylated form of mtFabH, were clearly detected. The more acidic spot was excised from the acrylamide gel and analyzed by mass spectrometry after tryptic digestion. The MS/MS spectrum of the corresponding triply charged ion unambiguously confirmed the presence of a single phosphate group on the threonine residue 45, consistent with the phosphoacceptor identified in *in vitro* phosphorylated mtFabH (Fig. S1). Whether the phosphorylation of mtFabH remains constant or is a growth phase-dependent phenomenon is presently not known and requires investigation. In addition, it would be particularly interesting to monitor the dynamics of mtFabH phosphorylation in cultures growing under different conditions (temperature, low oxygen concentration, etc.).

Overall, these results clearly indicate that (i) mtFabH is phosphorylated *in vivo*; (ii) two dominant forms of mtFabH co-exist in *M. bovis* BCG, a nonphosphorylated and a monophosphorylated form; and (iii) mtFabH is phosphorylated on a unique residue (Thr^{45}) both *in vitro* and *in vivo*.

Localization of Thr^{45} on the mtFabH Structure—In order to understand the effect of Thr^{45} phosphorylation on the enzymatic behavior of mtFabH, we examined the location of this residue with respect to the substrate binding site and the catalytic residues, taking advantage of the available three-dimensional structures of mtFabH (28, 40–42). As Fig. 5 illustrates, solvent-exposed Thr^{45} is situated more than 20 Å away from the catalytic cysteine at the surface of mtFabH. Thus, it is unlikely that a phosphoryl group on Thr^{45} will have a noticeable effect on the catalytic reaction mechanism. However, in the structure of apo-mtFabH (Protein Data Bank entries 1HZP (40) and 1M1M (28)), Thr^{45} is seen in close proximity ($\leq$6 Å) to residues Arg^{46} and Trp^{42}. Both residues play important roles in binding coenzyme A, the donor substrate in the ketoacyl synthase reaction; in the CoA-bound structure of *E. coli* FabH (Protein Data Bank entry 1HNJ (43)), Arg^{46} coordinates the pyrophosphate, whereas Trp^{42} stacks on top of the adenine base. The latter interaction is also seen in the methyl-CoA disulfide-bound structure of mtFabH (Protein Data Bank entry 2EFT (42)). Indeed, we showed in a previous study that alanine mutations on these sites markedly reduced (although they not completely abrogate) β-ketoacyl synthase activity (28), underscoring a role of these residues in substrate binding. A phosphate group on Thr^{45} would offer an alternative salt bridge interaction for Arg^{46}, which would require this residue to assume a rotamer conformation incompatible with simultaneous binding to the CoA-pyrophosphate. Likewise, a phosphorylated Thr^{45} may influence the conformational freedom of Trp^{42}, impeding the stacking interaction of this residue with the adenine base. Moreover, it is conceivable that phosphorylation of Thr^{45} affects the interaction of mtFabH with the acyl carrier protein AcpM, which also must bind in the vicinity of the narrow substrate binding channel of mtFabH. Thus, the structural analysis suggests that phosphorylation of Thr^{45} may affect substrate binding and/or protein-protein interactions with other FAS-II partners (44). Therefore, the condensing activities of wild-type mtFabH with that of point mutants, Thr^{45} to alanine and aspartic acid, were assessed and compared *in vitro*. These point mutations prevent mtFabH phosphoryla-

tion and introduce a mimic of the negative charge of a phosphate group, respectively.

Enzymatic Activity Is Negatively Regulated in an mtFabH_T45D Mutant—The reaction catalyzed by the mtFabH homodimer initiates with the binding of an acyl-CoA generated by FAS-I, following transacylation of the acyl chain of the bound CoA to the catalytic Cys^{122}, allowing the CoA moiety dissociation from the protein and the malonyl-AcpM to bind to the active site liberated by CoA (40). The malonyl-AcpM is then decarboxylated to form a carbanion, which in turn reacts with the Cys^{122}-bound acyl group to generate the final product of the reaction, the β-ketoacyl-AcpM. We thus assessed whether phosphorylation could have an influence on the different activities characterizing mtFabH (*i.e.* the condensation, transacylation, and malonyl-AcpM decarboxylation).

Following a strategy that has been successfully used to demonstrate that regulation of Wag31 via phosphorylation is important during active cell growth in mycobacteria (45), we expressed and purified mtFabH_T45D, the phosphorylation mimic. Previous studies have shown that acidic residues, such as Asp or Glu, qualitatively recapitulate the effect of phosphorylation with regard to functional activity (46). Condensing activities of mtFabH_WT, mtFabH_T45A, and mtFabH_T45D were determined and compared. The overall condensing activities of mtFabH_WT and mtFabH_T45A were similar (Fig. 6*A*). This suggests that Thr^{45}, unlike its neighboring residue Arg^{46} (28), does not play a critical role in the condensation reaction, consistent with its surface-exposed localization (Fig. 5). In contrast, mtFabH_T45D expressed a 42% reduced activity with respect to mtFabH_WT activity. Since the Asp mutant mimics constitutive phosphorylation of mtFabH, these results suggest that the introduction of a negative charge at position 45 has a negative impact on the condensing activity of the enzyme. Phosphorylation may rather modulate mtFabH on a fine tuned level rather than a strict on/off mechanism, as previously proposed for Arg^{46}, involved in the binding of acyl-CoA substrates (40), since decreased overall activity was observed in the mtFabH_R46A mutant (28).

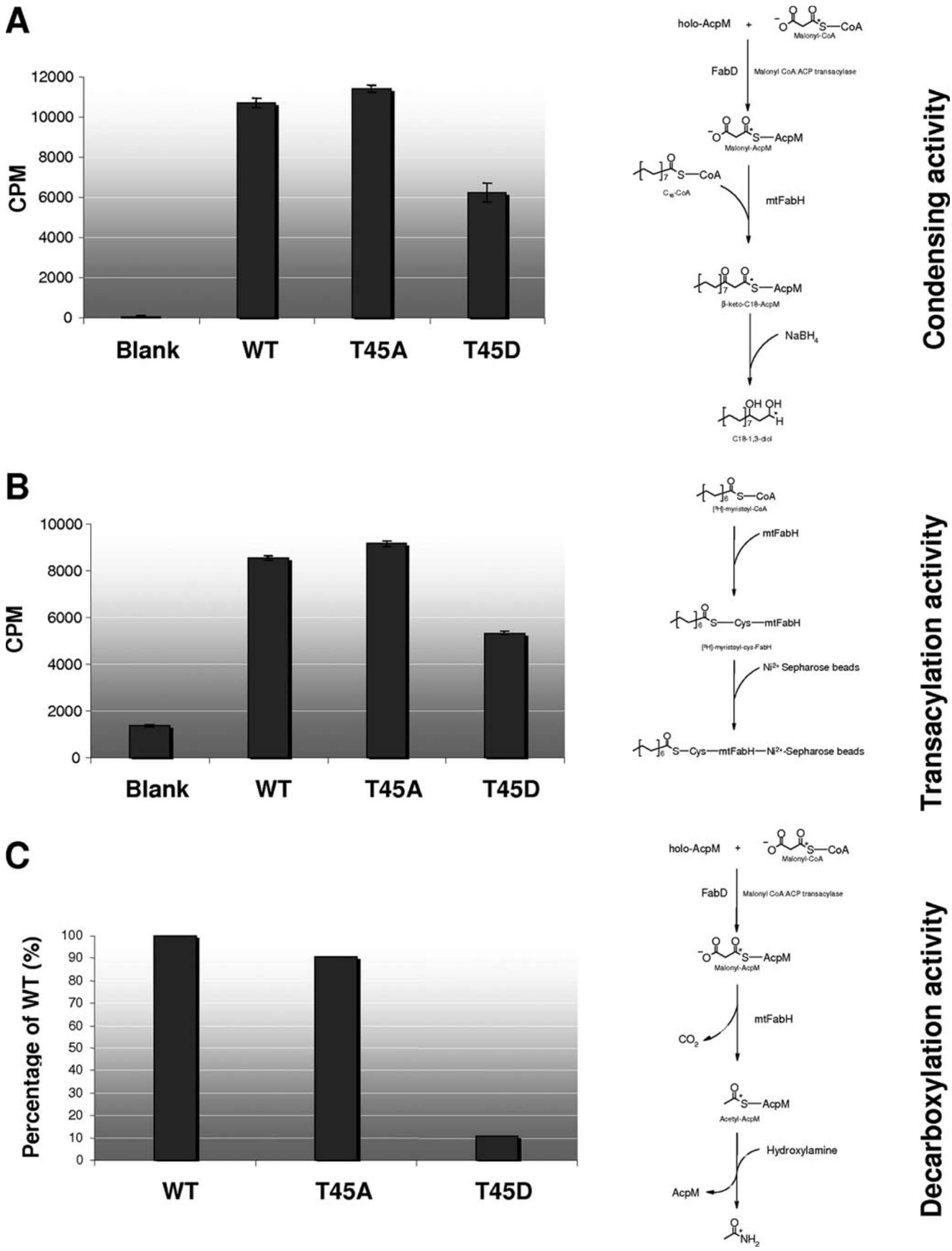
When the transacylation reaction was examined, both mtFabH_WT and mtFabH_T45A behaved similarly (Fig. 6*B*). However, as for the condensing activity, the mtFabH_T45D mutant exhibited a significant reduced transacylase activity compared with mtFabH_WT. As mentioned above, the presence of a negative charge affects part-reaction activity of mtFabH. These results are in favor of a model where the substitution of the neighboring Arg^{46} by Ala has a moderate effect on the transacylation activity of mtFabH (28).

In the malonyl-AcpM decarboxylation assay, the activity of mtFabH_WT was arbitrarily fixed at 100%. The substitution of Thr^{45} by Ala only slightly affects the decarboxylation activity (Fig. 6*C*). Remarkably, the decarboxylative activity of mtFabH_T45D was severely inhibited and was found to represent about 10% of the wild-type activity (Fig. 6*C*). As mentioned above, mimicking the phosphorylation state of Thr^{45} fits properly with the overall effects observed in the case of R46A mutant, in which the decarboxylation activity was almost completely abrogated (28). As with the R46A mutation, the condensation activity of the T45D mutant protein was less affected than observed in the decarboxylation activity. This can be attributed to the enzymatic state of the protein during the decarboxylation assays, upon which no acyl chain is bound to the active site Cys, which may change the overall decarboxylation activity of the protein, since the two part-reaction assays are not true kinetic representations of the condensation activity.

DISCUSSION

The β-ketoacyl-ACP synthase III of *M. tuberculosis* (mtFabH) links the FAS-I and FAS-II systems, catalyzing the condensation of FAS-I-derived acyl-CoAs with malonyl-AcpM (28, 32, 40). The first step of the reaction, catalyzed by a mtFabH homodimer, involves transfer of an acyl group from acyl-CoA to the enzyme active site. This is followed by a two-carbon acyl chain extension through a Claisen-type condensation with malonyl-AcpM. The resulting β-ketoacyl-AcpM product is then reduced to acyl-AcpM by the β-ketoacyl-AcpM reductase MabA (47–49), followed by a dehydratation step carried on by the newly identified β-hydroxyacyl-AcpM dehydratases Rv0635-7 (FabZ′, FabZ, and FabZ″, respectively), also known as HadABC (50, 51), and reduced by the enoyl-AcpM reductase InhA, the primary target of isoniazid (52, 53). In this study, we identified mtFabH as a substrate of STPKs in mycobacteria. Phosphorylation of this enzyme occurs both *in vitro* and *in vivo* on a single and identical residue, Thr^{45}. It is noteworthy that mtFabH interacts with several STPKs *in vitro*, suggesting that it may be regulated by multiple environmental signals. Identification of the Thr^{45} phosphoacceptor allowed us to assess the role of phosphorylation on the different activities characterizing mtFabH (condensation, transacylation, and malonyl-AcpM decarboxylation). The comparative analysis of enzymatic activities in mtFabH and Thr^{45} point mutants clearly demonstrates that phosphorylation on residue 45 is able to exert a marked influence on the activity of this enzyme and thus supports the hypothesis of regulation through STPK-mediated phosphorylation. Based on structural constraints, a direct effect on catalysis can be ruled out. However, the structural parameters are compatible with the assumption that Thr^{45} phosphorylation regulates transacylation, decarboxylation, and condensing activities through affecting substrate binding. How precisely this regulatory effect comes to bear remains to be elucidated. Interactions of a phosphorylated Thr^{45} with adjacent residues Arg^{46} and Trp^{42} are likely to affect binding of CoA. Nonetheless, the enzymatic data suggest that the latter effect is less important than the interference with binding of AcpM, demonstrated by the 90% reduction of decarboxylation activity in mtFabH_T45D compared with wild type. Indeed, the parallel effects on decarboxylation of the $Arg^{46} \rightarrow$ Ala mutation in Ref. 28 and that of the $Thr^{45} \rightarrow$ Asp mutation observed here hint that the Thr^{45}-Arg^{46} surface patch constitutes a critical element of the interaction with AcpM. Together, these data suggest that phosphorylation may fine tune the interactions of mtFabH with the components of the FAS-II system.

We have previously shown that mtFabD, KasA, and KasB are substrates of *M. tuberculosis* STPKs (24). There is now a strong body of evidence that several FAS-II components are phosphorylated and that STPK-dependent phosphorylation can induce either positive or negative signaling to the different interconnected FAS-II complexes. Indeed, a model based on co-exist-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CPM
12000
10000
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WT
T45A
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holo-AcpM
Malonyl-CoA
FabD
Malonyl CoA:ACP transacylase
Malonyl-AcpM
mtFabH
β-keto-C18-AcpM
NaBH4
C18-1,3-diol
Condensing activity
B
CPM
10000
8000
6000
4000
2000
0
Blank
WT
T45A
T45D
mtFabH
Cys—mtFabH
Ni2+ Sepharose beads
Cys—mtFabH—Ni2+-Sepharose beads
Transacylation activity
C
Percentage of WT (%)
100
90
80
70
60
50
40
30
20
10
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WT
T45A
T45D
holo-AcpM
Malonyl-CoA
FabD
Malonyl CoA:ACP transacylase
Malonyl-AcpM
mtFabH
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Acetyl-AcpM
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AcpM
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Decarboxylation activity

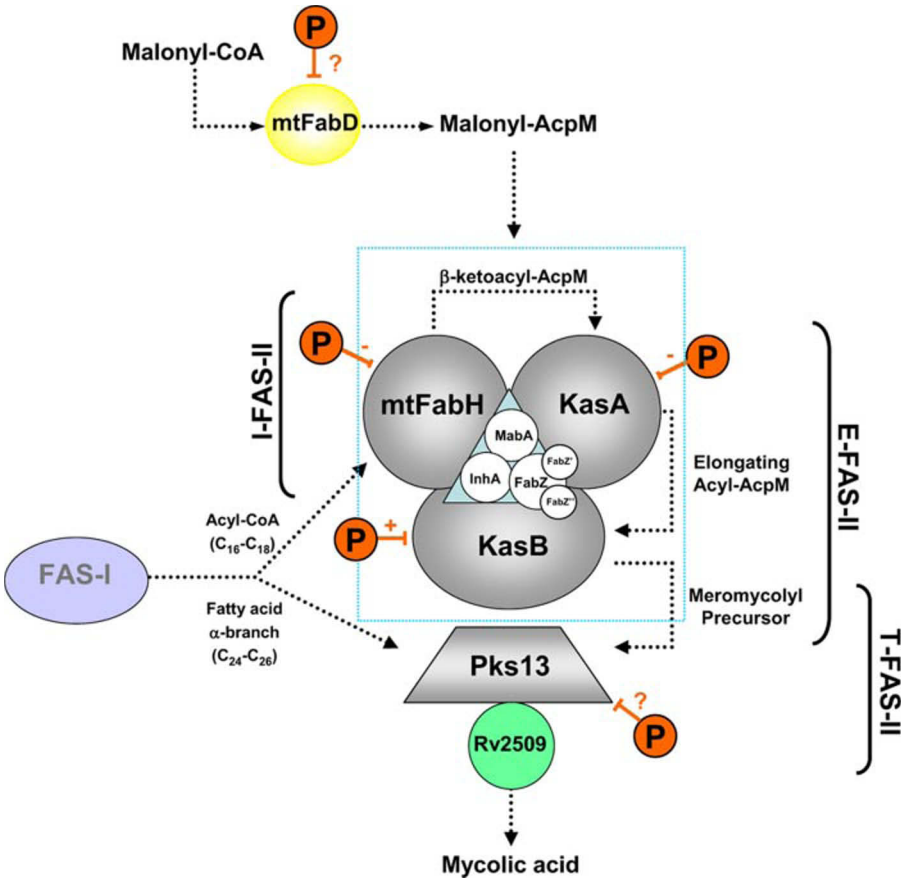

FIGURE 7. **Proposed regulatory role of condensase phosphorylation in mycolic acid initiation, elongation and termination.** Mycolic acid biosynthesis is initiated via *de novo* biosynthesis of C_{16-26}-CoA by the mycobacterial FAS-I and further extended to the meromycolate precursor by FAS-II. FAS-I provides both the initiation primer (C_{16}-CoA) for FAS-II and the C_{24-26} fatty acid utilized by the Claisen-type condensing enzyme Pks13 (belonging to the T-FAS-II system) during the formation of the nonreduced mycolic acid. The initiation primer is utilized by mtFabH (a feature of the I-FAS-II system), forming a β-ketoacyl-AcpM product, which is further modified via the reductive cycle of FAS-II performed by MabA, FabZ, FabZ′, FabZ″, and InhA. The derived aliphatic acyl-AcpM then is used as the substrate of the condensing enzymes KasA and KasB of the E-FAS-II systems. KasA is involved in the elongation of the meromycolate precursors that are further extended to their full lengths by KasB. The condensing enzymes are shown in *gray*. Experimental evidence has demonstrated phosphorylation of all four condensases. Phosphorylation can either activate (+) or inhibit (−) the condensing activity. A *question mark* indicates that the effect of phosphorylation on the enzyme activity has not yet been investigated.

ence of multiple interconnected FAS-II systems has been proposed (44). It predicts the occurrence of four FAS-II systems responsible for the initiation, elongation, and termination steps of the mycolic acid pathway, each system characterized by a common enzyme core along with a specific condensase (Fig. 7). The "initiation FAS-II" (I-FAS-II) is characterized by the condensase mtFabH, which converts an acyl-CoA from FAS-I to form β-ketoacyl-AcpM. Upon completion of the FAS-II reductive cycle, this acyl-AcpM derived product primes the "elongation-1 FAS-II" (E1-FAS-II) characterized by the condensase KasA. E1-FAS-II elongates the acyl-AcpM to longer chain acyl-AcpM products, which are then channeled into the "elongation-2 FAS-II" (E2-FAS-II), comprising KasB that completes the meromycolate precursor synthesis (35, 36). This acyl product is further modified by various modification enzymes to form the mature meromycolate (31). Finally, the polyketide synthase Pks13 (54) of the "termination FAS-II" system catalyzes the condensation of the meromycolate (C_{56}) with the α-branch (C_{26}) (produced by FAS-I) to generate oxo-mycolic acids, which are reduced to mature mycolates by Rv2509 (55). Our results support a model in which STPK-dependent phosphorylation of the condensases can induce either positive or negative signaling to the I-FAS-II and E-FAS-II complexes. In support of this view, our preliminary experiments also demonstrated that, in addition to KasA, KasB, and mtFabH, the remaining condensase Pks13 is also phosphorylated by *M. tuberculosis* STPKs (data not shown). Thus, the differential expression of the mycobacterial STPKs in response to stress/environmental conditions may directly influence the phosphorylation profile of all four condensases, which in turn may modulate the different interconnected FAS-II systems. It is, therefore, tempting to hypothesize that phosphorylation controls critical enzymatic steps of the FAS-II pathway and subsequently regulation of mycolic acid biosynthesis in order to promote adaptation to environmental changes and survival within the infected host.

It is noteworthy that overexpression of mtFabH in *M. bovis* BCG leads to an unusual smooth texture of the colony morphology and is accompanied by alterations in complex lipid composition, including a decrease in phthiocerol dimycocerosate (PDIM) synthesis (32). These methyl-branched fatty acids are formed by unique enzymes, which utilize acyl-CoA primers rather than ACP-bound primers (56). Because mtFabH uses long chain acyl-CoA primers and not acyl-ACP primers (32), overexpression of this enzyme in mycobacteria may decrease

FIGURE 6. **Comparative enzymatic activities of wild-type and mutant mtFabH proteins in KAS-III and part-reaction assays.** Each part-reaction step is depicted by a *schematic representation beside* the corresponding *bar charts*. *, the position of the radiolabeled carbon. In each schema, the final product is shown. *A*, whole condensing activity. The assays were performed in triplicate on two separate protein preparations, and activity was assessed by the scintillation counting method using AcpM and a palmitoyl-CoA primer. *B*, the extent of transacylation activity of mtFabH and its mutants was determined by quantifying the binding of [^{3}H]myristoyl-CoA to the protein after incubation by liquid scintillation counting of protein-bound radiolabeled acyl chains captured on Ni^{2+}-charged chelating Sepharose beads. Assays were performed in triplicate on two separate protein preparations. *C*, the malonyl-AcpM decarboxylation activity was obtained from a direct densitometric reading from the autoradiogram normalized to the activity of mtFabH_WT with AcpM and expressed as a percentage, as described under "Experimental Procedures" and by Brown *et al.* (28). *WT*, wild type.

the pool of acyl-CoA primers available for PDIM and other related complex lipid biosynthetic pathways. In addition, complex lipids, such as PDIMs, have been shown to interact with host cells and participate in the virulence and pathogenesis of *M. tuberculosis* (22, 57). This suggests that the expression level of mtFabH or regulation of its activity by phosphorylation may also be a determinant for *M. tuberculosis* virulence by controlling the pool of acyl-CoAs available for complex lipid synthesis. Supporting the view of a connection between FAS-II and PDIM biosynthetic pathways, a recent study conducted by Kruh *et al.* (58) described a novel interaction between KasA and polyketide modules involved in the biosynthesis of PDIM. These observations suggest that regulation of the activity of condensing enzymes, such as mtFabH (and KasA), by phosphorylation may directly participate in the control of both the FAS-II and the PDIM pathways. Further studies are needed to ascertain this hypothesis.

Although we provide here, for the first time, evidence that phosphorylation of mtFabH occurs *in vivo* in mycobacteria, more work is required to address whether it influences the production of mycolic acids (and PDIM synthesis) during *in vivo* growth and eventually if it participates in mycobacterial survival/virulence within the infected host. Using specialized transduction, it is now feasible to derivate isogenic strains of *M. tuberculosis* differing by single point mutations in the genome (53). This technology would be particularly useful to transfer T45A or T45D point mutations that will either prevent or mimic constitutive phosphorylation of mtFabH in *M. tuberculosis* and investigate whether phosphorylation of mtFabH represents an important physiological event *in vivo*.

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