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Cyclic AMP-dependent Protein Lysine Acylation in Mycobacteria Regulates Fatty Acid and Propionate Metabolism*[S]

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Background: KATmt is the first identified cAMP-regulated protein lysine acetylase in mycobacteria.
Results: KATmt acylates fatty acyl CoA ligases *in vivo* in a cAMP-dependent manner, thus regulating their activity.
Conclusion: Mycobacteria utilize KATmt to regulate the metabolic pool of acetyl and propionyl CoA.
Significance: We provide novel paradigms for linking cAMP signaling and fatty acid metabolism in mycobacteria.

Acetylation of lysine residues is a posttranslational modification that is used by both eukaryotes and prokaryotes to regulate a variety of biological processes. Here we identify multiple substrates for the cAMP-dependent protein lysine acetyltransferase from *Mycobacterium tuberculosis* (KATmt). We demonstrate that a catalytically important lysine residue in a number of FadD (fatty acyl CoA synthetase) enzymes is acetylated by KATmt in a cAMP-dependent manner and that acetylation inhibits the activity of FadD enzymes. A sirtuin-like enzyme can deacetylate multiple FadDs, thus completing the regulatory cycle. Using a strain deleted for the KATmt ortholog in *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG), we show for the first time that acetylation is dependent on intracellular cAMP levels. KATmt can utilize propionyl CoA as a substrate and, therefore, plays a critical role in alleviating propionyl CoA toxicity in mycobacteria by inactivating acyl CoA synthetase (ACS). The precision by which mycobacteria can regulate the metabolism of fatty acids in a cAMP-dependent manner appears to be unparalleled in other biological organisms and is ideally suited to adapt to the complex environment that pathogenic mycobacteria experience in the host.

Tuberculosis remains one of the leading causes of death because of an infectious disease. The causative agent of tuberculosis, *Mycobacterium tuberculosis*, is harbored by almost 30% of the population of the world. However, most infected people do not develop the disease. Thus, a delicate balance is maintained between the pathogen and a healthy host that does not show signs of illness. This balance can tilt toward the pathogen if the host is coinfected with HIV or has a weakened immune response because of dietary or environmental conditions, and understanding the factors that disturb the equilibrium is, therefore, important.

A number of virulence factors in *M. tuberculosis* allow it to evade the natural immune response of the host, establish infection, and remain persistent over many years in a dormant state. The availability of the genome sequence of *M. tuberculosis* served as an important molecular tool in dissecting the roles of individual genes in pathogenesis (1). We found some years ago that a number of genes involved in cAMP (3′,5′-cAMP) synthesis (adenylyl cyclases) were encoded in many mycobacterial genomes (2, 3). Cyclic AMP, a universal second messenger, provides a means by which the pathogen can communicate with and hijack host signaling within macrophages during early infection (4, 5). Indeed, it has been reported that deletion of one of the 16 adenylyl cyclase genes in *M. tuberculosis* results in attenuation of virulence (6), pointing toward an important role for cAMP/adenylyl cyclases in *M. tuberculosis* pathogenesis.

Elevated cAMP levels are, however, also seen in non-pathogenic strains of mycobacteria, indicating that cAMP has important roles to play in the basic biology of mycobacteria (7). We therefore set out to identify targets of cAMP in mycobacteria and focused on proteins that contained a cyclic nucleotide binding domain identified earlier and characterized in many eukaryotic proteins and the bacterial transcription factor cyclic AMP receptor protein, CRP (8, 9). We identified unique proteins (MSMEG_5458 and Rv0998) from *Mycobacterium smegmatis* and *M. tuberculosis*, respectively, that consist of a cyclic nucleotide binding domain fused to an acetyltransferase domain. We demonstrated that these proteins act as cAMP-regulated protein lysine acetyltransferases (10) and showed that a universal stress protein is a substrate in *M. smegmatis*. Rv0998, which we call KATmt, required the presence of cAMP to show acetyl transferase activity. Recent biophysical and structural analyses revealed the dramatic conformational change that occurs in these proteins on cAMP binding that allows the acetylation of its protein substrates (11, 12).

Orthologs of KATmt are found in all mycobacteria, including *Mycobacterium leprae*, indicating that this protein must have

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an important role to play in these organisms. The fusion of a cyclic nucleotide binding domain along with an acetyltransferase domain in a single gene product is not found in any other prokaryotic or eukaryotic genome sequenced thus far, implying that the direct regulation of protein acetylation by intracellular cAMP levels in mycobacteria is unique to this genus. In this study, we have identified substrates of KATmt and show for the first time that a number of mycobacterial fatty acyl Co-A ligases are acylated *in vitro* and *in vivo* by KATmt. We thus perhaps uncover some of the first mechanisms downstream of adenylyl cyclases and cAMP within mycobacteria during establishment and, possibly, persistence in the host macrophage.

EXPERIMENTAL PROCEDURES

Bioinformatic Analysis—Six amino acids surrounding the acetylated Lys residue in universal stress proteins were used as a seed sequence for BLASTP analysis against the predicted *M. tuberculosis* protein database (NCBI). Proteins that were identified were then inspected manually to confirm that the Lys residue was preceded by a small amino acid and followed by a few small hydrophobic residues (13). Proteins that were identified were then subjected to further analysis using Predmod (13).

Multiple sequence alignment of the 34 FadDs[2] was done using ClustalW (14, 15), and a phylogenetic tree was generated using Molecular Evolutionary Genetics Analysis software (16).

Cloning, Expression, and Purification of Proteins—The list of primers used for PCR and mutagenesis are provided in supplemental Table S1. All clones were generated and verified by sequencing (Macrogen, South Korea). Cloning strategies are available on request. A clone of FadD13 containing a mutation at Lys-517 was provided by Prof. A. K. Tyagi, University of Delhi, India. Proteins were expressed in the *E. coli* BL21 endo$^-$ strain following induction with 500 μM isopropyl 1-thio-β-D-galactopyranoside for 20 h at 16 °C or for 3 h at 37 °C, as described earlier (10).

For MSMEG_5175 (sirtuin) and MSMEG_5175$_{H104Y}$, cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 8.2), 500 mM NaCl, 20% glycerol, 5 mM 2-mercaptoethanol, 2 mM PMSF, and 1 mM benzamidine. Washes were done with buffer containing 50 mM Tris-Cl (pH 8.2), 500 mM NaCl, 20% glycerol, 5 mM 2-mercaptoethanol, and 20 mM imidazole. The proteins were eluted with buffer containing 50 mM Tris-Cl (pH 8.2), 500 mM NaCl, 20% glycerol, 5 mM 2-mercaptoethanol, and 300 mM imidazole. KATmt was expressed in the *E. coli* SP850 strain on induction using 500 μM 1-thio-β-D-galactopyranoside for 20 h at 16 °C as described earlier (10).

Western Blotting—Samples were electrophoresed on a 12% SDS-polyacrylamide gel and transferred to a PVDF membrane (Immobilon-P, Millipore). FadD13 polyclonal antibody was generated in the laboratory and used at a dilution of 1:5000. Acetyl lysine antibody (Cell Signaling Technology, Inc.) was used at a dilution of 1:2500. Horseradish peroxidase-conjugated secondary antibody (GE Healthcare) or light chain-specific antibody (Jackson ImmunoResearch Laboratories) were used and detected by enhanced chemiluminescence according to the instructions of the manufacturer (GE Healthcare).

Mass Spectrometric Analysis—Recombinant purified protein samples after *in vitro* acylation were treated with 400 ng of trypsin for 5 h in solution. The peptide mixtures were then analyzed by liquid chromatography and electrospray tandem mass spectrometry (LC-ESI MS/MS). The samples were analyzed on an HCT-Ultra ETD-II ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany), coupled to an Agilent 1100 HPLC. The LC separation was performed on a reverse-phase C18 column at a flow rate of 0.2 ml/min using a H_2O-acetonitrile (with 0.1% formic acid) solvent system under a gradient of 90% H_2O to 90% acetonitrile over a period of 60 min. The mass spectral analysis was performed under auto-MS/MS conditions, with the three most intense ions from each scan being subjected to CID fragmentation. The fragmentations were carried out inside the ion trap through the collision of He gas with the ions of interest, which were excited kinetically by an increased resonance amplitude of the dipolar field, with a value typically set between 1–2 V. The scan speed was set at 26,000 m/z s-1. The m/z ranges for MS detection and MS/MS precursor ion selection were set from 300–1800 and 500–1400, respectively.

In Vitro Deacylation Assay—FadDs (2 μg) were acetylated or propionylated by KATmt (200 ng). Samples were then directly treated with sirtuin$_{WT}$ or sirtuin$_{H104Y}$ (500 ng) in the presence or absence of 1 mM NAD^+. Deacetylation reactions were carried out at 37 °C in buffer containing 25 mM Tris-Cl (pH 8.2), 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, and 100 ng/μl BSA for 1 h. Whenever necessary, deacetylation reactions were also performed in the presence of 5 mM nicotinamide, a known sirtuin enzyme inhibitor. Reactions were stopped by boiling in SDS sample buffer and analyzed by Western blotting with acetyl lysine antibody and chemiluminescent HRP substrate (Immobilon, Millipore).

FadD Assays—The activities of acylated or non-acylated FadDs were assessed using an *in vitro* fatty acyl CoA synthetase assay with ^{14}C-labeled palmitic acid as the substrate. The purified FadD protein (1 μg) was initially incubated with 10 mM DTT for 30 min at 37 °C prior to its use. Subsequently, purified enzymes were incubated with 50 μM acetyl CoA or propionyl CoA, 25 mM Tris-Cl (pH 7.5), 1 mM cAMP, and 300 ng of KATmt for 30 min at 37 °C to acylate the FadDs in a reaction volume of 10 μl.

Fatty acyl CoA synthetase assays were performed in 50 mM Tris-Cl (pH 8.0), 100 μM ^{14}C-palmitic acid, 5 mM ATP, and 2.5 mM $MgCl_2$ in a 10-μl reaction volume and, wherever applicable, CoA (2 mM). After prewarming the reaction mixture at 30 °C for 15 min, purified protein (acetylated or non-acetylated) was added to a final reaction volume of 20 μl and incubated for 5 min at 30 °C. The reactions were quenched with 5% acetic acid and directly spotted on silica gel TLC plates. Products were resolved in *n*-butanol/acetic acid/water (15:5:8) at 4 °C. The radioactive bands on TLC were detected using a phosphoimager (Bass 1800, Fuji) after 12 h of exposure.

Mycobacterial Cell Culture and Preparation of Cell Lysates—*Mycobacterium bovis* BCG was grown in Middlebrook's 7H9 broth supplemented with 0.2% glycerol, 10% ODAC (oleic acid,

[2] The abbreviations used are: FadD, fatty acyl CoA synthetase; ACS, acyl CoA synthetase; BCG, Bacillus Calmette-Guérin; CID, collision-induced dissociation.

albumin, dextrose and catalase) and 0.03% tyloxapol (Sigma-Aldrich). Initially the cultures were maintained in volume of 5 ml in tissue culture flasks (T-25 cm^2, Thermo Scientific) without shaking until the optical density reached 1. This culture was then used as inoculum for large-scale cultures with an initial optical density of 0.1. Whenever necessary, hygromycin (50 μg/ml) was used. For carbon utilization experiments, bacteria were grown in 7H9 medium containing 0.03% tyloxapol and glucose (0.2%). Sodium propionate was used at a concentration of 10 mM. For SDS stress, cells were grown until the optical density was 0.8 and then treated with 0.05% SDS for 1.5 h at 37 °C.

For preparation of whole cell lysates, cells were broken by bead beating in buffer containing 10 mM Tris-Cl (pH 7.5), 100 mM NaCl, 10% glycerol, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 3 mM nicotinamide, and 1 μM trichostatin A. Nonidet P-40 and deoxycholate to final concentrations of 1% and 0.5%, respectively, were added to the lysate and mixed continuously at 4 °C for 2 h, after which samples were centrifuged for 30 min at 4 °C at 13,000 × *g*. Supernatants were collected, and protein was estimated using a Micro BCA protein assay kit (Invitrogen).

Measurement of cAMP in M. bovis BCG—Cells were centrifuged at 13,000 × *g*; washed with buffer containing 10 mM Tris-Cl (pH 7.5), 0.89% NaCl, and 0.05% Tween 80 (TBST); and then the pellet was resuspended in 0.1N HCl and the suspension was boiled for 10 min. Aliquots of the lysate were taken for estimation of cAMP by radioimmunoassay.

Immunoprecipitation of FadD13—Antibodies to FadD13 were generated in rabbits. Anti FadD 13 IgG was purified using a T-Gel purification kit (Thermo Scientific Pierce) according to the instructions of the manufacturer. For preclearing, cell lysates (300 μg) were incubated with normal rabbit IgG for 1 h at 4 °C. Protein G beads were added to the lysates and incubated for an additional 30 min. The beads were removed by centrifugation, and the precleared supernatant was interacted with FadD13 IgG overnight at 4 °C. Protein G beads were added to the lysates and incubated for an additional 2 h. The beads were pelleted at 4 °C and washed thrice with buffer containing 10 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 0.1% Triton X-100 and twice with TBS (10 mM Tris-Cl (pH 7.5), 100 mM NaCl). The beads were boiled in SDS sample buffer and subsequently analyzed by SDS-PAGE and Western blotting.

RNA Isolation and Reverse Transcriptase—PCR Cells were harvested and resuspended in 300 μl of Tri reagent (Sigma-Aldrich) prewarmed to 65 °C. Approximately 50 mg of glass beads (with a diameter of 465–600 μm, Sigma-Aldrich) were added, and bead beating was carried out for 90 s with 30-s intervals at 4800 rpm using a BeadBeater (BioSpec Products). The lysate was mixed by pipetting and followed by centrifugation at 12,000 × *g* for 10 min at 4 °C. The supernatant was treated with chloroform (200 μl) followed by centrifugation at 14,000 × *g* for 15 min at 4 °C. The RNA was precipitated with 200 μl of isopropyl alcohol, and the RNA pellet was dissolved, quantified, and treated with RNase-free DNase (1–5 units). RNA (2 μg) was used for reverse transcription using 200 units of reverse transcriptase (MBI Fermentas, Canada). The transcript levels of *BCG_1055 (KATbcg)*, *FadD13*, and *sirtuin* were assessed using primer pairs Rv0998RTFWD and Rv0998RTRVS, Rv3089RTFWD and Rv3089RTRVS, and Rv1151cRTFWD and Rv1151cRVS, respectively (supplemental Table S1).

Generation of Δ*KATbcg strain—M. bovis* BCG with a deletion of *KATbcg* was generated using allelic exchange using a specialized transducing phage, phAE87 (provided by Prof. Sabine Ehrt, Weill Cornell Medical College). A region ~650 bp upstream of *KATbcg* was amplified using the Rv0998upFWD and Rv0998upRVS primers (5′ amplicon) (supplemental Table S1). Similarly, a fragment ~650 bases downstream of *KATbcg* was amplified using the Rv0998downFWD and Rv0998downRVS primers (3′ amplicon) (supplemental Table S1), and fragments were cloned into the pBKSII (+) vector. Clones were confirmed by sequencing. The AgeI-SphI fragment from pBKS-Rv0998upKO was ligated to similarly digested pJSC284-loxP (provided by Prof. Sabine Ehrt, Weill Cornell Medical College) to generate pJSC284-loxP-Rv0998upKO. The BamHI-HindIII fragment from pBKS-Rv0998downKO was ligated into the similarly cut pJSC284-loxP-Rv0998upKO plasmid to generate the pJSC284-loxP-Rv0998updownKO plasmid. The isolated phage DNA was ligated using 5 Weiss units of DNA ligase at 16 °C for 12 h. After ligation, the phage DNA was subjected to PacI digestion. The digested phage DNA was then ligated to PacI-digested pJSC284-loxP-Rv0998updownKO. The packaging of the ligated phage DNA was carried out in the laboratory of Dr. Apoorva Bhatt, School of Biosciences, University of Birmingham. Approximately 5–20 μg phagemid DNA was electroporated into electrocompetent *M. smegmatis* cells and plated on 7H10 agar plates. Plaques appeared 2–3 days later at 30 °C. The phages were amplified, and phage DNA was isolated to check the presence of pJSC284-loxP-Rv0998updownKO by PCR. The desired phage was transduced into exponential phase *M. bovis* BCG, and they were screened for the absence of KATbcg by genomic PCR using the Rv0998FWD and Rv0998RVS primers (supplemental Table S1). They were further confirmed by Southern blotting and RT-PCR.

To construct the Δ*KATbcg* strain complemented with the *KATbcg* gene driven by its own promoter, the Rv0998upFWD and Rv0998RVS primers (supplemental Table S1) were used to amplify 650 bp upstream of *KATbcg*. The PCR product was digested with BamHI and EcoRI and ligated into similarly digested pBKSII (+) to generate pBKS-opromRv0998. The NotI-EcoRI fragment from pBKS-opromRv0998 was cloned into similarly digested pMV306 (a gift from Prof. Sabine Ehrt, Weill Cornell Medical College) to generate pMV306-opromRv0998, which was electroporated in the Δ*KATbcg* strain. Positive integrants carrying the required insert were screened by colony PCR and validated by Southern hybridization and RT-PCR.

RESULTS

Multiple Substrates for KATmt—A loose consensus for predicting lysine residues that have a propensity for acetylation has been identified (13), and a stretch of small or flexible amino acid directly preceding the acetylated lysine residue is often observed (17, 18). Therefore, we used six amino acids surrounding the acetylated Lys residue in universal stress proteins as a seed sequence for BLASTP analysis against the predicted

M. tuberculosis protein database. Proteins that were identified were then inspected manually to confirm that the Lys residue was preceded by a small amino acid and followed by a few small hydrophobic residues, as seen in the universal stress protein sequence. Sixty proteins were thus identified and were then subjected to further analysis using Predmod to determine their propensity for acetylation (13). Eighteen proteins had a significant score (supplemental Fig. 1*a*), suggesting that these proteins may be acetylated by KATmt.

We selected seven proteins, cloned their genes, and expressed all of them in *E. coli* (supplemental Fig. 1*b*). Rv2948c (FadD22), Rv0270 (FadD2), Rv3089 (FadD13), and Rv0166 (FadD5) are acyl CoA synthetases, Rv1007c is predicted to be a methionyl tRNA synthetase, Rv1633 is an enzyme involved in nucleotide excision repair, and Rv0640 is a 50 S ribosomal protein, L11. We also cloned Rv3667 (ACS), which has been shown earlier to be acetylated by the *M. smegmatis* KAT MSMEG_5458 (19). Purified proteins were treated individually with KATmt in the presence of cAMP and acetyl CoA (Fig. 1*a*). The Mtb 50 S ribosomal protein L11, Rv0640, was acetylated as purified, probably by an *E. coli* protein acetyltransferase, and showed no further increase in acetylation in the presence of KATmt. ACS was acetylated by KATmt in a cAMP-dependent manner in agreement with earlier results. Although FadD22 (Rv2948c), FadD2 (Rv0270), FadD5 (Rv0166), and FadD13 (Rv3089) were acetylated in a cAMP-dependent manner, Rv1633 and Rv1007c were not acetylated by KATmt. Therefore, we were able to identify KATmt substrates through sequence analysis with some degree of confidence, and, as shown below, the predictive efficiency was better for the family of FadDs we chose to study in greater detail.

The biochemical and structural features of FadD13 have been described recently (20–22). We have therefore used it in further studies as a representative substrate for KATmt. We mapped the site of acetylation in FadD13 using LC-MS/MS and identified Lys-487 as the acetylated residue (Fig. 1*b*). In agreement with the LC-MS/MS data, a mutant FadD13 (K487A) showed no acetylation in the presence of KATmt, cAMP, and acetyl CoA (Fig. 1*c* and supplemental Fig. 1*c*), indicating that Lys-487 was the sole site of acetylation.

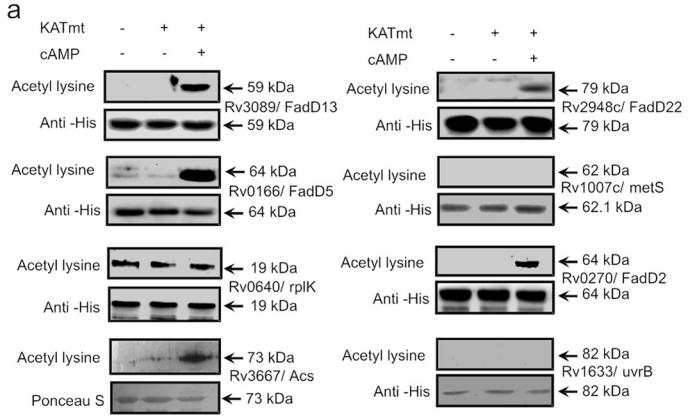
Strikingly, all tested substrates for KATmt (FadD22, FadD2, FadD5, and FadD13) belonged to the fatty acyl CoA synthetase family of proteins that activate lipids prior to their utilization in metabolic pathways (23). The site of acetylation of FadD22, FadD2, and FadD5 was the Lys residue present at a position equivalent to Lys-487 in FadD13. Because this lysine residue is conserved in FadD enzymes, it was of interest to determine whether all of these proteins were substrates for KATmt. We therefore cloned and expressed all 34 FadD enzymes in *E. coli*. Twenty-two proteins were purifiable from *E. coli*, although the remainder were localized to inclusion bodies (supplemental Fig. 1*d*). We tested all 22 proteins for their ability to be acetylated by KATmt in a cAMP-dependent manner, and, as shown in Fig. 1*d*, eight enzymes were acetylated. The site of acetylation in these proteins, as analyzed by LC-MS/MS (supplemental Fig. 1*e*) was the Lys residue present at an equivalent position to Lys-487 in FadD13 (Fig. 1*e*). LC-MS/MS also confirmed that the other 14 FadD enzymes were not acetylated (because peptides resulting from tryptic cleavage following the C-terminal Lys residue could be detected, supplemental Fig. 1*f*).

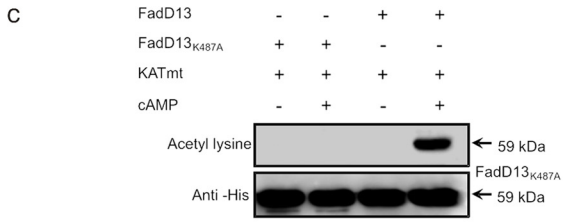
We aligned the sequences of the FadDs that were acetylated as well as those that were not (Fig. 1*e*). We observed the conservation of motifs GG*X*NX_4E*X*E/D, E/DX_7E/D, K/A*X*P, and PX_4GK in the C-terminal domain sequences of FadDs that were acetylated, whereas non-acetylated FadDs showed conservation in some, but not all, of these motifs together. This implies a concerted influence of a set of conserved residues in the C-terminal domain of FadDs, rather than just residues in the immediate proximity of the acetylated lysine residue that determine substrate specificity. Acetylated FADs clustered together on a dendrogram built on a sequence alignment of the full-length proteins (Fig. 1*f*), whereas the non-acetylated enzymes branched separately. Inspection of the dendrogram and sequences of the non-purifiable enzymes allowed us to predict whether a protein could be acetylated or not by KATmt (supplemental Fig. 1*g*). Because FadDs 1, 11, and 15 do not have the PX_4GK motif and FadDs 21 and 33 lack the K/A*X*P, E/DX_7E/D, and GG*X*NX_4E*X*E/D motifs, we predict that they may not be acetylated by KATmt. On the other hand, FadDs 8, 3, 14, and 36 retain most of the important motifs for acetylation and, therefore, could be substrates for KATmt. Manual inspection of the sequences of FadD16 and FadD9 revealed that they lack the PX_4GK motif, which includes the acetylated Lys, at the C terminus and are, therefore, unlikely to be acetylated.

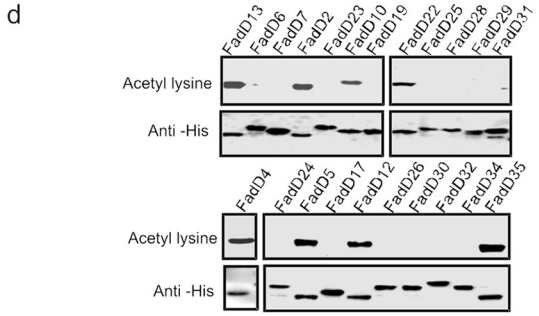
Deacetylation of FadDs by a Mycobacterial Sirtuin-like Enzyme—Lysine acetylation is a reversible process (24, 25), and deacetylation can be carried out by the sirtuin class of deacetylases. Sirtuin-like enzymes can be identified in bacterial genomes (25, 26), and one such enzyme, the product of the Rv1151c gene, has been shown to deacetylate ACS (19). We cloned and purified the ortholog of Rv1151c from *M. smegmatis*, MSMEG_5175 (sirtuin) (the former being more difficult to purify, supplemental Fig. 2, *a* and *b*), and monitored its ability to deacetylate FadD13 (supplemental Fig. 2*c*). Sirtuins are NAD^+-dependent enzymes, and a critical His residue is present at the active site, which, on mutation to Tyr, inactivates enzyme activity (27). As seen in Fig. 2*a*, sirtuin was able to deacetylate FadD13 only in the presence of NAD^+, whereas the sirtuin$_{H104Y}$, lacking the catalytic residue, showed no activity either in the presence or absence of NAD^+. Inclusion of nicotinamide, an inhibitor of sirtuin-like enzymes, prevented the deacetylation of FadD13 (28).

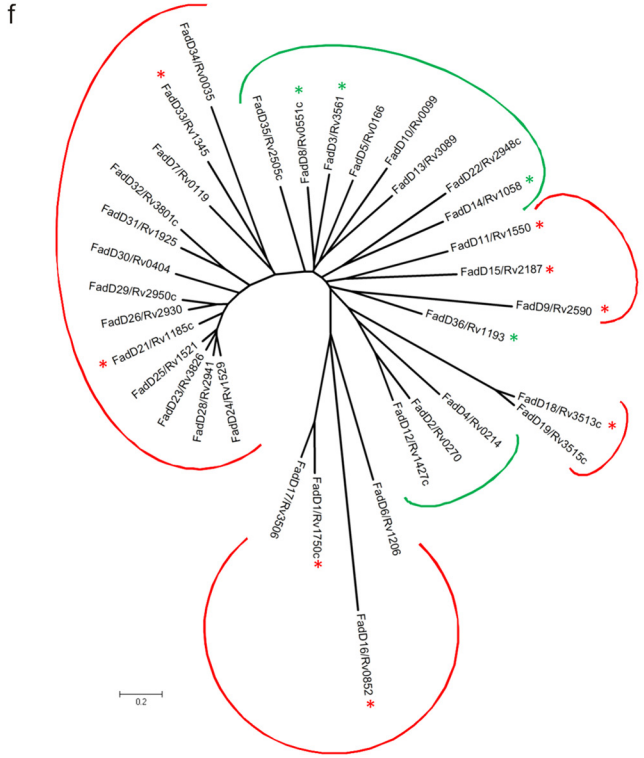
We then proceeded to test whether sirtuin could deacetylate all the FadD enzymes that we have shown previously to be acetylated by KATmt. As shown in Fig. 2*b*, all enzymes could be deacetylated, some more efficiently than others, perhaps being reflective of sirtuin substrate specificity or the presence of additional domains following the adenylation domain, as seen in FadD22 (29). These results, therefore, indicate that the state of FadD acetylation in the cell could be dynamically regulated by KATmt and sirtuin.

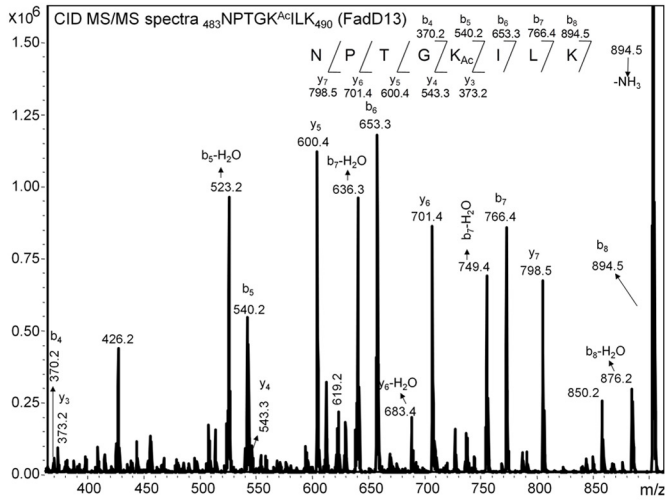
Acylation Inhibits the AMP-ligase Activity of FadD Enzymes—Lys-487 in FadD13 has been shown to be critical for the binding of ATP prior to the formation of the adenylated fatty acid, which is subsequently converted to the CoA derivative (20, 22). Therefore, reversible acetylation of this critical catalytic residue











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FadD10/Rv0099     RSSEMIICGGVNIAPDEVDRIAEGVSGVREAACYEIPDEEFGALVGLAVVASA---ELDE 479
FadD22/Rv2948c    RADDTEVIGGVNVDPREVERLIIEDEAVAEAAVVAVRESTGASTLQAFLVATSGA-TIDG 444
FadD35/Rv2505c    RIKDLVVRGGENISPREIEELLHTHPDIVDGHVIGVPDAKYGEELMAVVKLRNDAPELTI 496
FadD4/Rv0214      RRHHMIISGGVNIYPQEAENLLVAHPKVLDAAVFGVPDDEMGQRVMAAVQTVDSA-DAND 489
FadD12/Rv1427c    RDDEMIVSGGENIYPIEVEKTLATHPDVAEAAVIGVDDQQYGQRLAAFVVLKPGV-SATP 490
FadD2/Rv0270      RDDEMIVSGGENVFPAEVEDLISGHPDVVEAAAIGVDDKEFGARLRAFVVKKPGA-DLDE 518
FadD5/Rv0166      RKKDMIISGGENIYCAELENVLASHPDIAEVAVIGRADEKWGEVPIAVAAVTND--DLRI 486
FadD13/Rv3089     RLKDMIISGGENVYPAEIESVIIGVPGVSEVAVIGLPDEKWGEIAAAIVVADQN--EVSE 454
                  *    : ** *:  * :       : :     :  .

FadD10/Rv0099     SAARALKHTIAARFRRESEPMARPSTIVIVTDIPRTQSGKVMRASLAAAA--------TA 531
FadD22/Rv2948c    SVMRDLHRGLLNRLSA----FKVPHRFAVVDRLPRTPNGKLVRGALRKQSPTKPIWELSL 500
FadD35/Rv2505c    E---RLREYCMGRIAR----FKIPRYLWIVDEFPMTVTGKVRKVEMRQQA---------- 539
FadD4/Rv0214      QFAGELLAWLRDRLSH----FKCPRSIAFEPQLPRTDTGKLYKSGLVEKV---------- 535
FadD12/Rv1427c    E---TLKQHVRDNLAN----YKVPRDIAVLDELPRGITGKILRTELQSRV---------- 533
FadD2/Rv0270      D---TIKQYVRDHLAR----YKVPREVIFLDELPRNPTGKVLKRELRKL----------- 560
FadD5/Rv0166      E---DLGEFLTDRLAR----YKHPKALEIVDALPRNPAGKVLKTELRLRY---------- 529
FadD13/Rv3089     Q---QIVEYCGTRLAR----YKLPKKVIFAEAIPRNPTGKILKTVLREQY---------- 497
                  .   :     .:         * . .  :*    **: . :

FadD10/Rv0099     DKARVVVRG---------------------------------------------------- 540
FadD22/Rv2948c    TEPGSGVRAQRDDLSASNMTIAGGNDGGATLRERLVALRQERQRLVVDAVCAEAAKMLGE 560
FadD35/Rv2505c    ---LEYLRGQQ---------------------------------------------------- 547
FadD4/Rv0214      ---SV----------------------------------------------------------- 537
FadD12/Rv1427c    ---GS----------------------------------------------------------- 535
FadD2/Rv0270      ---------------------------------------------------------------- 560
FadD5/Rv0166      ---GACVNVERRSASAGFTERRENRQKL----------------------------------- 554
FadD13/Rv3089     ---SATVPK------------------------------------------------------ 503

FadD34/Rv0035     ----------RQDEVFIVHGENRFPYDIEFIIRGESE-QHRTKVACFGVN---------- 467
FadD32/Rv3801c    ----------RIKDLVIIDGRNHYPQDLECTAQESTKALRVGYAAAFSVPANQLPQTVFD 539
FadD30/Rv0404     ----------RMKDLIIQDGVNHYPDDIETTVKE----FTGGRVAAFSVS---------- 499
FadD31/Rv1925     ----------RIADLLTIDGRNHYPQDIEATAAEASPMVRRGYITAFTVPAS-------- 541
FadD29/Rv2950c    ----------RIKELLVVDGANHYPEDIEATIQE----ITGGRVVAIAVP---------- 533
FadD26/Rv2930     ----------RIKDLLIVDGRNHYPDDIEATIQE----ITGGRAAAIAVP---------- 497
FadD28/Rv2941     ----------RIKDLLIVYGRNHSPDDIEATIQE----ITRGRCAAISVP---------- 495
FadD23/Rv3826     ----------RIKDLLIVYGRNHSPDDIEATIQE----ITRGRCAAIAVP---------- 495
FadD24/Rv1529     ----------RIKDLLIVYGRNHAPDDIEATIQE----ITSGRCAAIAVP---------- 500
FadD25/Rv1521     LSEGELFIMGRIKDLLIVYGRNHSPDDIEATIQT----ISPGRCAAIAVS---------- 499
FadD19/Rv3515c    ----------RGSVSINSGGEKVYPEEVEAALKGH---PDVFDALVVGVP---------- 466
FadD7/Rv0119      ----------RIKELINRGGEKISPERVEGVLASH---PNVMEAAVFGVP---------- 456
FadD6/Rv1206      ----------RLGDTFRWKGEIVATTQVEAALASD---QTVEECTVYGVQ---------- 493
FadD17/Rv3506     ----------RLGDWMRVDGENLGTAPIERVLMRY---PDATLVAVYPVP---------- 423
                          *   .  * :  . :*              *

FadD34/Rv0035     -------------ERV-VVVLESPLDSIIDKAEADRLR-------CQVVAATGLQLDEL 505
FadD32/Rv3801c    DSHAGLKFDPEDTSEQL-VIVGE-RAAGTHKLDHQPIVDDIR----AAIAVGHGVTVRDV 593
FadD30/Rv0404     ----------DDGVEHL-VIAAEVRTEHGPDKVTIMDFSTIKRLVVSALSKLHGLHVTDF 548
FadD31/Rv1925     --------DGDDRNQRL-VIIAE-RAAGTSRSDPRPALDAIR----AAVCNRHGLSVADL 587
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FadD19/Rv3515c    ----------DPRYGQQV-AAVVQARPGCRPSLAELDSFV------RSEIAGYK---VPR 176
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FadD17/Rv3506     ----------DPVVGDQV-MAALVLAPGTKFDADKFRAF-------LTEQPDLGHKQWPS 465

FadD34/Rv0035     ITVRRGAIPTTTSGKLKRRAVAQAYRDGTLPR-LATHAWTADPDSAPKTTRSSLEGAH-- 562
FadD32/Rv3801c    LLVSAGTIPRTSSGKIGRRACRAAYLDGSLRSGVGSPTVFATSD---------------- 637
FadD30/Rv0404     LLVPPGALPKTTSGKISRAACAKQYGANKLQR-VATFP--------------------- 585
FadD31/Rv1925     SFLPAGAIPRTTSGKLARQACRAQYLSG---R-LGVH---------------------- 620
FadD29/Rv2950c    VMVAPGSIPVTTSGKVRRSASVERYLHHEFSR-LDAMA--------------------- 619
FadD26/Rv2930     VLVSPGSIPITTSGKIRRSACVERYRSDGFKR-LDVAV--------------------- 583
FadD28/Rv2941     VLVAPGSIPITTSGKVRRGACVEQYRQDQFAR-LDA----------------------- 580
FadD23/Rv3826     VLVAPGSIPITTSGKVRRAECVKLYRHNEFTR-LDAKPLQASDL--------------- 584
FadD24/Rv1529     VLVSPGSIPITTSGKIRRAQCVQLYRRREFTR-LDA----------------------- 584
FadD25/Rv1521     VLVSPGSIPITTSGKIRRAQCVELYRQDEFTR-LDA----------------------- 583
FadD19/Rv3515c    SLWFVDEVKRSPAGKPDYRWAKEQTEARPADDVHAGHVTSGG----------------- 548
FadD7/Rv0119      SFQEASGLPHTAKGSLDRRAVAERFGHSV------------------------------ 525
FadD6/Rv1206      FVRVVGSLAHTTTFKSRKVELRNQAYGADIEDPLYVLAGPDEGYVPYYAEYPEEVSLGRR 594
FadD17/Rv3506     YVRVSAGLPRTMTFKVIKRQLSAEGVA------CADPVWPIRR----------------- 502
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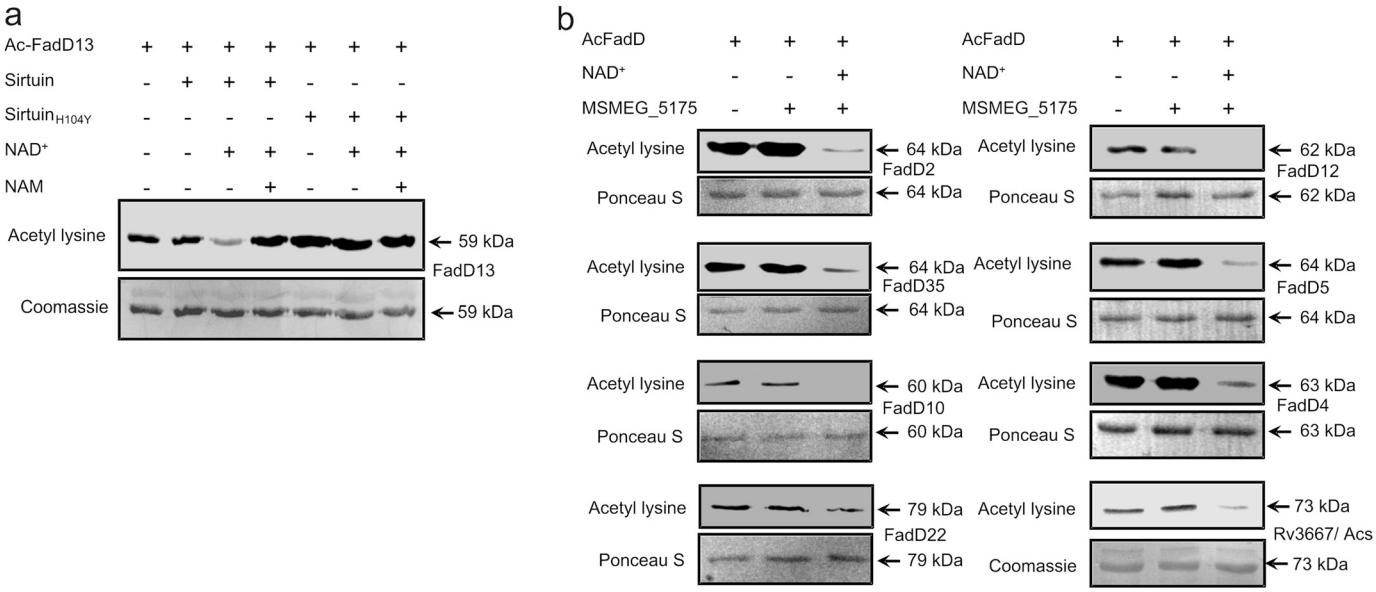

FIGURE 2. **Deacetylation of FadDs by sirtuin.** *a*, FadD13 (2 μg) was acetylated by KATmt (200 ng) at 37 °C for 30 min and then incubated with sirtuin (500 ng) or sirtuin$_{H104Y}$ in the presence of NAD$^+$ (1 mM) for 1 h at 37 °C. Nicotinamide (*NAM*, 2 mM) was added to the reaction mixture as indicated. Western blot analysis was performed with acetyl lysine antibody (*upper panel*) followed by Coomassie Brilliant Blue staining of the blot (*lower panel*). *b*, two micrograms of the indicated FadDs were acetylated using KATmt and then deacetylated using sirtuin in the presence of NAD$^+$ (1 mM), as described previously, followed by Western blotting with acetyl lysine antibody.

and the equivalent Lys residues in the other FadD enzymes could regulate their biochemical activities.

The substrates of all FadDs from mycobacteria have not been identified. However, three of the FadD enzymes (FadD13, FadD5, and FadD2) that were putative substrates for KATmt could charge palmitic acid to form the CoA derivative (Fig. 3*a*), but to different extents. As shown in Fig. 3*a*, acetylation of all these enzymes by KATmt prevented the formation of palmitoyl-AMP. Given the conservation of the Lys residue that is acetylated and its critical role in the formation of the fatty acyl-AMP conjugate, we predict that the activities of all the FadD enzymes that serve as substrates for KATmt are inhibited following acetylation.

In addition to acetyl CoA, some protein acetyl/propionyl transferases can also utilize propionyl CoA, resulting in the propionylation of proteins (30). Using FadD13 as a substrate, we demonstrated propionylation of the same Lys residue in FadD13 that was acetylated (Fig. 3*b*), resulting in inhibition of the activity of FadD13 in a manner similar to that seen on acetylation (*c*). We then tested whether the presence of propionyl CoA affected the utilization of acetyl CoA by KATmt. As shown in Fig. 3*d*, increasing concentrations of propionyl CoA in the presence of acetyl CoA inhibited the acetylation of FadD13, presumably leading to its propionylation and a reduction in immunoreactivity with the acetyl lysine antibody. Therefore, the relative levels of acetyl CoA or propionyl CoA in the cell could determine whether a substrate of KATmt is acetylated or propionylated.

Propionylated FadD13 showed a low cross-reactivity with the acetyl lysine antibody used for Western blot analysis (Fig. 3*d*, *last lane*). We exploited this to determine whether sirtuin was able to depropionylate FadD13. As shown in Fig. 3*e*, we could detect efficient depropionylation of FadD13 by the sirtuin enzyme, thus providing evidence for a more general mechanism of protein acylation and deacylation in mycobacteria and modulation of activities of substrate proteins.

In Vivo Acetylation of FadD13 in Mb BCG—The activity of KATmt is precisely regulated by cAMP, as revealed by biochemical assays (Fig. 1*a*). We therefore extended these *in vitro* biochemical studies to monitor cAMP-dependent acetylation and deacetylation of KATmt substrates *in vivo*. Cyclic AMP levels in mycobacteria are modulated depending on environmental conditions and growth media (7, 31). The sequences of KATmt and its ortholog in *M. bovis* BCG, BCG_1055 (KATbcg), are identical, as are the sequences of FadD13 and its ortholog BCG_3114. *M. bovis* BCG grown in glycerol-containing media had 10-fold higher cAMP levels as compared with growth in glucose-containing media (Fig. 4*a*). We therefore monitored the acetylation status of FadD13 in cells grown under these conditions where intracellular levels of cAMP differed. Cells grown in glycerol showed robust acetylation of FadD13, whereas cells grown in glucose showed no acetylated

FIGURE 1. **Cyclic AMP-dependent acetylation of FadDs by KATmt.** *a*, two micrograms of purified proteins were incubated in the presence of KATmt (300 ng), cAMP (1 mM), and acetyl CoA (50 μM), followed by Western blotting with acetyl lysine antibody and anti-His antibody. *b*, CID MS/MS spectrum of the acetylated tryptic peptide $_{483}$NPTGK^{Ac}ILK$_{490}$ of FadD13. The observed fragment ions (b and y ions) are marked on the spectrum and also summarized schematically. *c*, FadD13$_{K487A}$ (2 μg) was incubated with KATmt, cAMP, and acetyl CoA, followed by Western blotting with acetyl lysine antibody and anti-His antibody. *d*, *in vitro* acetylation of indicated FadDs (500 ng) was performed as indicated, followed by Western blotting with acetyl lysine antibody and anti-His antibody. *e*, multiple sequence alignment for FadDs that are acetylated by KATmt (*upper panel*) and those that are not (*lower panel*). Conserved residues are highlighted by *colored boxes*. The *red box* (*lower panel*) highlights the variation in the highly conserved K/AXP motif. The acetylated lysine is marked with a *green dot* in both panels. *f*, dendrogram of full-length sequences of 34 FadDs. The acetylated and non-acetylated FadDs are highlighted with *green/red arcs*, respectively. Using the same color code and an *asterisk*, the potential of 12 FadDs to be acetylated by KATmt is predicted.

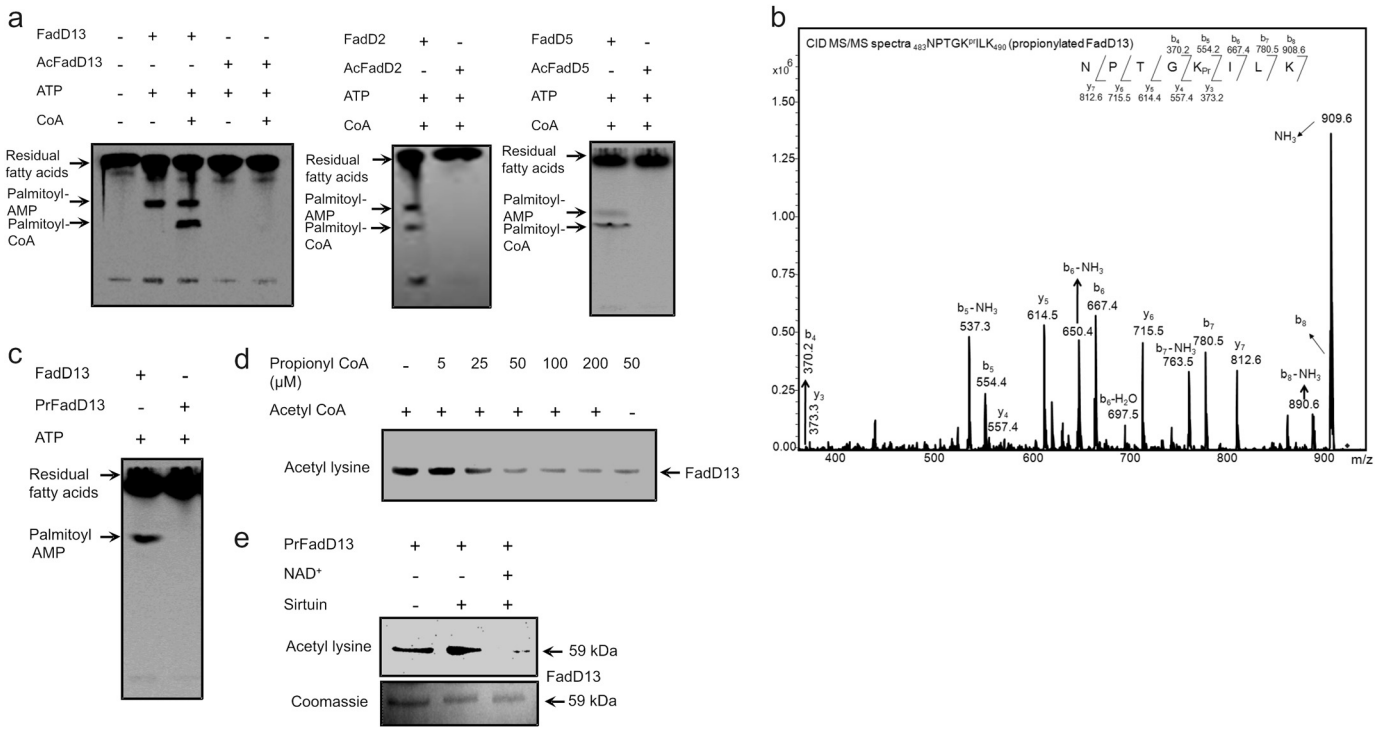

FIGURE 3. **Inactivation of FadDs by KATmt following acylation.** *a*, FadD or acetylated FadD were incubated with ^{14}C-palmitic acid, ATP (5 mM), and CoA (2 mM), as indicated, at 30 °C for 5 min. Fatty acid migrated on the TLC with a relative mobility of 0.96. Palmitoyl AMP and palmitoyl CoA had Rf values of 0.6 and 0.51, respectively. *b*, CID MS/MS spectrum of the singly charged species of the propionylated tryptic peptide $_{483}$NPTGK^{Pr}ILK$_{490}$ of FadD13 (*m/z* 926.4) The signature b and y ions present in the spectrum are marked, and a schematic summary of all the b and y ions obtained is shown. *c*, FadD13 or propionylated FadD13 were incubated with ^{14}C-palmitic acid and ATP (5 mM), as indicated, at 30 °C for 5 min. *d*, assays were performed in the presence of varying concentrations of propionyl CoA with a fixed amount of acetyl CoA (50 μM), and acetylated FadD13 was analyzed using Western blot analysis. *e*, two micrograms of the indicated FadD13 was propionylated using KATmt (200 ng, 30 °C, 30 min) and then deacetylated using sirtuin (500 ng) in the presence of NAD$^+$ (1 mM) at 37 °C for 1 h, followed by Western blotting with acetyl lysine antibody.

FadD13 (Fig. 4*b*), even though levels of the sirtuin ortholog BCG_1212c and KATbcg were equivalent (*c*). Therefore, there appears to be a correlation between the intracellular levels of cAMP and the activity of KATbcg *in vivo*, resulting in the subsequent acetylation of its substrates.

We treated cells grown in glucose with 0.05% SDS, a procedure that, as we have shown earlier, results in an increase in intracellular cAMP in *M. smegmatis* (7). Indeed, cAMP levels were elevated in *M. bovis* BCG cells more than10-fold following SDS treatment (Fig. 4*d*). As shown in Fig. 4*e*, FadD13 was now found to be acetylated following SDS treatment. The levels of both sirtuin and FadD13 remained unaltered in the SDS-treated cells (Fig. 4*f*), whereas a 4-fold increase in the KATbcg transcript on SDS treatment was observed. This, together with the increase in intracellular cAMP seen on SDS treatment, could now result in activation of KATbcg and increased acetylation of FadD13 (Fig. 4*d*).

We next generated a strain of *M. bovis* BCG that was deleted for KATbcg (Δ*KATbcg*, supplemental Fig. 3*a*). Approximately 650 bp upstream of KATbcg, which would contain the promoter, were cloned along with the gene and integrated into the knockout strain to generate a complemented strain. The genomic integrity of these strains was confirmed by Southern blot analysis (supplemental Fig. 3*b*). The transcript for *KATbcg* was absent in the knockout strain, and transcript levels were restored to wild-type levels in the complemented strain (supplemental Fig. 3*c*). We grew cells in glycerol and monitored the acetylation status of FadD13 in these strains. As seen in Fig. 4*g*, FadD13 was not acetylated in the knockout strain grown either in glycerol- or glucose-containing media. Acetylation was restored on complementation of KATbcg in glycerol-containing media to the extent seen in the wild-type strain. These results, therefore, demonstrate that FadD13, and presumably other substrates of KATbcg, are acetylated exclusively by KATbcg and not by any other protein acetyltransferase that may be present in the cell. As a consequence, there is a specific and exquisite regulation of cAMP-dependent protein acetylation in mycobacteria, brought about by this unique acetyltransferase.

Deletion of KATbcg in Mb BCG Leads to Compromised Growth on Propionate-containing Media—Mycobacteria are sensitive to high levels of intracellular propionyl CoA, presumably because of the formation of toxic propionyl CoA metabolites (32–34). ACS from *M. tuberculosis* is equally efficient in converting acetate or propionate to their CoA derivatives (35). We hypothesized that inhibition of the activity of ACS by acylation via KATbcg may be an important way of regulating the accumulation of propionyl CoA in the cell. We first confirmed that ACS could be propionylated by KATmt in the presence of cAMP (Fig. 5*a*). We then grew wild-type, Δ*KATbcg* and the Δ*KATbcg* strain complemented with a wild-type copy of KATbcg in media containing either propionate or glucose as the sole carbon source. Growth of the *KATbcg* deletion strain was not compromised in media containing glucose (where acetyl CoA formation by glycolytic pathways would be predom-

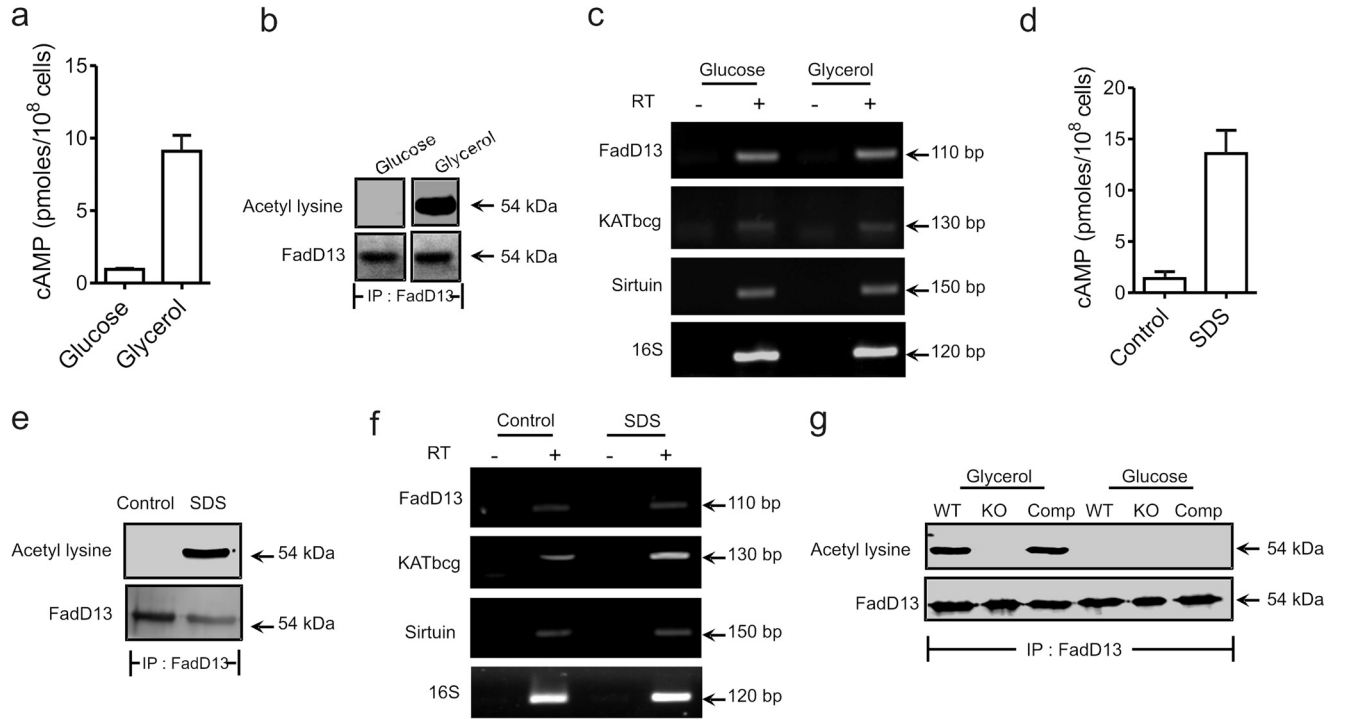

FIGURE 4. **FadD13 is acetylated by KATmt in a cAMP-dependent manner.** *a*, intracellular cAMP levels during growth of wild-type *M. bovis* BCG in medium containing 0.2% glycerol or 0.2% glucose as sole carbon source. Data shown are mean ± S.E. of duplicate determinations, with the experiment being repeated thrice. *b*, whole cell lysates from *M. bovis* BCG grown in 7H9 media containing 0.2% glycerol or 0.2% glucose were prepared. FadD13 was immunoprecipitated and subjected to Western blot analysis with acetyl lysine antibody and FadD13 antibody. *c*, semiquantitative RT-PCR analysis of BCG_3114 (FadD13), BCG_1055 (KATbcg), and BCG_1212c (sirtuin) from RNA prepared from wild-type *M. bovis* BCG grown in 0.2% glucose or 0.2% glycerol as sole carbon source. 16 S primers were used as a normalization control. *d*, wild-type *M. bovis* BCG was treated with 0.05% SDS for 1.5 h, and the intracellular cAMP level was measured. All data shown represent the mean ± S.E. of duplicate determinations, with each assay being performed thrice. *e*, whole cell lysates was prepared from SDS-treated cells. FadD13 was immunoprecipitated and subjected to Western blot analysis with acetyl lysine antibody and FadD13 antibody. *f*, semiquantitative RT-PCR analysis of FadD13, KATbcg, and sirtuin from RNA prepared from SDS-treated cells. 16 S was used for normalization. *g*, whole cell lysates from WT, knockout (*KO*), and complemented (*Comp*) strains grown in 7H9 media containing 0.2% glycerol or 0.2% glucose were prepared. FadD13 was immunoprecipitated and subjected to Western blot analysis with acetyl lysine antibody and FadD13 antibody.

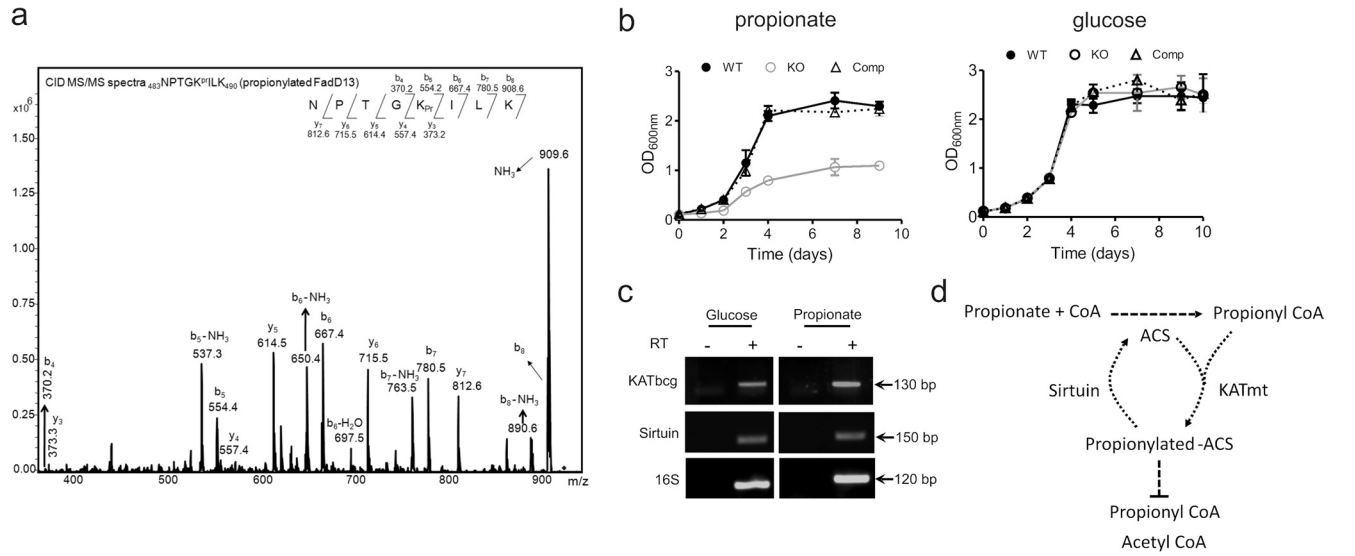

FIGURE 5. **Propionylation and inactivation of ACS by KATmt.** *a*, CID MS/MS spectrum of the singly charged species of the propionylated tryptic peptide $_{614}$SGK^{Pr}IMR$_{620}$ of ACS (*m/z* 747.5). The signature b and y ions present in the spectrum are marked, and a schematic summary of all the b and y ions obtained is shown. *b*, growth analysis of the WT, knockout (*KO*), and complemented (*Comp*) strains in 7H9 media containing 0.2% propionate as the sole carbon source. Shown are the A_{600} values from three independent biological replicates. *c*, semiquantitative RT-PCR analysis of KATbcg and sirtuin from RNA prepared from wild-type *M. bovis* BCG grown in 0.2% glucose or 0.2% propionate as the sole carbon source. 16 S primers were used as a normalization control. *d*, schematic depicting enzymatic inactivation of ACS because of propionylation by KATmt.

inant), but growth in propionate was reduced dramatically (Fig. 5*b*). Growth could be restored to levels seen for the wild-type strain following complementation with KATbcg. Although cAMP levels in wild-type cells grown in either propionate (2.7 ± 0.7 pmol/ml/optical density) or glucose (Fig. 4*a*) were equivalent, the levels of *KATbcg* mRNA were increased signif-

icantly in cells grown in propionate (5.1 ± 0.25-fold as estimated by real-time quantitative RT-PCR, Fig. 5*c*). However, the mRNA levels of the sirtuin ortholog BCG_1212c remained similar. It is therefore likely that the high levels of intracellular propionyl CoA, which is found in cells grown in propionate as the sole carbon source, necessitate the propionylation of ACS by KATbcg, resulting in its inactivation. This would prevent the further build up of propionyl CoA, thereby alleviating toxicity (Fig. 5*d*).

DISCUSSION

In this study, we have identified multiple substrates for cAMP-dependent protein acylation in *M. tuberculosis* and, for the first time, demonstrated the relevance of this posttranslational modification *in vivo*. Protein acetylation in bacteria occurs widely, but mycobacteria appear to represent the first and so far the only case where acylation is directly regulated by cAMP. The presence of a sirtuin-like enzyme completes the machinery required for modulating protein lysine acylation *in vivo*, thus allowing mycobacteria to regulate the activities of various proteins on the basis of intracellular levels of cAMP, acetyl CoA, propionyl CoA, and NAD^+. Such sophistication in directly regulating protein acylation has not been reported to date.

On the basis of a sequence alignment of acetylated proteins, we were able to recognize conserved amino acids not only in the vicinity of the modified Lys residue but also some distance upstream (Fig. 1*e*). This appears to be a general feature of all other acetyltransferases (36). A recent structural analysis of FadD13 shows that the Lys residue that is acetylated by KATmt is buried in the catalytic site of the protein (20). Thus, for it to be acetylated, a significant structural change needs to occur. Perhaps the conservation of residues some distance away from the acylated Lys residue may allow an initial recognition of a substrate by KATmt, thus determining the specificity of acylation and also resulting in the required conformational change to expose the Lys residue that is acetylated.

Reversible protein lysine acetylation regulates diverse protein properties, including DNA-protein interactions, protein-protein interaction, subcellular localization, stability, and enzymatic activity (37–43). We show here in mycobacteria that a variety of proteins serve as substrates for KATmt, indicating that multiple processes are likely to be affected by cAMP-dependent protein acetylation in these organisms as well. Of the multiple FadDs encoded in mycobacterial genomes, only a subset of them appears to be substrates for KATmt, emphasizing again the non-redundant properties of the FadD family of enzymes (Fig. 1*f*). Interestingly, none of the fatty acyl AMP ligases (FAALs), enzymes involved in adenylation of fatty acids prior to their modification by polyketide synthetases during mycobacterial cell wall synthesis (44), appear to be acetylated by KATmt. It is interesting to note that in eukaryotes, a number of mitochondrial metabolic enzymes are modified by acylation, including those involved in lipid β-oxidation (45, 46), reflective of what we see in mycobacteria.

The nutrients available to *M. tuberculosis* inside the host macrophage are restricted, and a number of studies have shown that fatty acids and cholesterol serve as the primary source of carbon (47–53). However, degradation of cholesterol and odd-chain fatty acids leads to the generation of propionyl CoA, which is toxic to these bacteria (32, 34). There are multiple mechanisms by which the toxicity of propionyl CoA can be alleviated. Isocitrate lyase can utilize propionyl CoA to form succinate and pyruvate (32, 49), propionyl-CoA carboxylase can generate methylmalonyl-CoA, which can enter the methylmalonyl pathway to produce succinyl CoA (33), and methylmalonyl CoA can also be used for the formation of mycobacterial cell wall lipids (54). We show here that propionyl CoA can also be utilized by KATmt for propionylation of proteins and, importantly, that this propionylation can be reversed by the sirtuin enzyme (Fig. 3*d*). We propose that this allows the selective modification of proteins, depending on the intracellular levels of propionyl CoA, acetyl CoA, cAMP, and NAD^+, the latter being required for catalysis by the sirtuin. The precision by which *M. tuberculosis* can this regulate its metabolism appears to be unparalleled and suited to the complex environment that it is likely to experience in the macrophage during infection and persistence in the granuloma.

Although growth in propionate-containing media did not dramatically increase intracellular levels of cAMP, it appears that the cell is able to enhance protein acetylation by increasing the levels of KATmt mRNA (Fig. 5*c*). The *M. tuberculosis* ACS has been biochemically characterized, and in contrast to other species, it can utilize both acetate and propionate equally efficiently to generate their CoA derivatives (19, 35). This appears to be an evolutionary adaptation of this enzyme that would allow the generation of propionyl CoA that can be used for a variety of cellular processes, specifically in mycobacteria. However, under conditions of high concentrations of propionyl CoA, KATmt can propionylate and, thereby, inactivate ACS (Fig. 5*d*), thus preventing the further increase in propionyl CoA levels, which would prove toxic to the cell.

The promiscuity of the KATmt in utilizing acetyl or propionyl groups for acylation make it particularly suited to be enlisted in homeostatic responses to counter environmental or nutritional conditions that lead to elevated levels of propionyl CoA. This acylation appears obligatory for the alleviation of the toxicity of propionyl CoA metabolites by acting as a feedback loop for regulating propionyl CoA levels in the cell, thereby accounting for the poor growth of the Δ*KATbcg* strain.

In conclusion, we propose that KATmt influences a number of pathways in mycobacteria. Increases in intracellular cAMP, brought about by the enhanced activity of adenylyl cyclases, would result in the activation of KATmt. Fatty acyl Co-A ligases that degrade lipids (*e.g.* FadD13) (22) regulate phenolic glycolipid synthesis (FadD22) (29, 55), have a role to play in epithelial invasion (FadD2) (56), recycling of mycolic acids (FadD5) (57), utilization of cholesterol (FadD3) (58), or virulence (FadD10) (59) are regulated by acylation in a cAMP-dependent manner. Enzymes involved in intermediary metabolism are also regulated by acetylation, including ACS. Because ACS generates acetyl CoA and propionyl CoA equally efficiently in mycobacteria, inhibition of its activity following acylation may have a critical role to play in alleviating the toxicity of high levels of propionyl CoA. All of these mechanisms appear to be critically dependent on the levels of intracellular cAMP and/or KATmt,

making KATmt an important contributor to mycobacterial physiology and, possibly, pathophysiology.

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