Post-Translational Acetylation of MbtA Modulates Mycobacterial Siderophore Biosynthesis*.

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Iron is an essential element for life, but its soluble form is scarce in the environment and is rarer in the human body. Mycobacterium tuberculosis (Mtb) produces two aryl-capped siderophores, mycobactin (MBT) and carboxymycobactin (cMBT), to chelate intracellular iron. The adenylylating enzyme MbtA catalyzes the first step of mycobactin biosynthesis in two half-reactions: activation of the salicylic acid as an acyl-adenylate and ligation onto the acyl carrier protein (ACP) domain of MbtB to form covalently salicylated MbtB-ACP. We report the first apo-MbtA structure from Mycobacterium smegmatis at 2.3 Å. We demonstrate here that MbtA activity can be reversibly, post-translationally regulated by acetylation. Indeed the mycobacterial protein acetyltransferase (Pat), Rv0998, specifically acetylates MbtA on lysine 546, in a cAMP-dependent manner, leading to enzyme inhibition. MbtA acetylation can be reversed by the NAD+-dependent deacetyltransferase, Rv1151c (DAc). Deletion of Pat and DAc genes in Mtb revealed distinct phenotypes for strains lacking one or the other genes at low pH and limiting iron conditions. This study establishes a direct connection between the reversible acetylation system Pat/DAc and the ability of Mtb to adapt in limited iron conditions, which is critical for mycobacterial infection.
which determine the localization of the siderophore: long-chain fatty acylated MBT is membrane-associated while cMBT contains short-chain dicarboxy-fatty acids and is more water-soluble and is secreted by the pathogen (13,14). Two MBT gene clusters, Mbt-1 and Mbt-2 (Fig. 1B), are composed of a mixture of nonribosomal peptide synthetase and polyketide synthase enzymes (15). Three specific enzymes, MbtA, MbtM (also known as FadD33) and MbtK are enzymes (15). Three specific enzymes, MbtA, peptide synthetase and polyketide synthase 1B), are composed of a mixture of nonribosomal chain dicarboxy-fatty acids and is more water-membrane-associated while cMBT contains short-siderophore: long-chain fatty acylated MBT is which determine the localization of the domain of MbtB (Fig. 1C) (12). FadD33 activates the long chain fatty acid before its transfer onto the central N-hydroxy lysine residue of the MBT core (16). MbtK is a GNAT N-acetyltransferase that transfers the long chain fatty acid onto the mycobactin core (16).

Iron-dependent transcriptional regulators, IdeR and HupB, control the transcription level of genes involved in iron metabolism including Mbt-1 and Mbt-2 in order to avoid excessive iron uptake, storage and toxic overload in the bacterial cell (17,18). Another level of MBT regulation was recently characterized as a post-translational acetylation of FadD33 by the protein lysine acetyltransferase (Pat) in Mycobacterium smegmatis (Msmeq) (19). Pat acetylates and inactivates FadD33 in a cAMP-dependent manner, which is then reversed by a NAD'-dependent Sirtuin-like deacetylase, DAC1, to restore FadD33 activity. Pat has been structurally characterized and consists of an N-terminal cAMP-binding domain fused to a C-terminal GCN5-related N-acetyl-transferase domain (GNAT) (20,21). The N-terminal domain allows Pat to be activated by the second messenger, cAMP, produced by the adenylate cyclase (AC) enzymes. The Mtb genome encodes a total of 16 ACs, which are sensitive to diverse environmental stimuli, including pH, hypoxia, fatty acids and carbon dioxide level (22). This complex response system allows mycobacteria to rapidly adapt to external changes by relaying this information, via a specific cAMP pathway, to downstream effectors.

In this study, we demonstrate that the first committed step in mycobactin biosynthesis, catalyzed by MbtA, can also be reversibly post-translationally acetylated by Pat with the loss of its enzymatic activity, and reactivated by DAC through deacetylation. Deletion of Pat and DAC genes in Mtb highlights the importance of those specific two genes for normal MBT production during iron starvation.

RESULTS

Cloning, Expression, and Purification of MbtA. To avoid solubility problems, Mtb and Msmeq N-terminal-His6-tagged MbtA were expressed at low temperature. Heterologous expression in E. coli gave good quantities of soluble MbtA. After purification by nickel affinity chromatography an apparent molecular weight of ~59 kDa was observed by SDS-PAGE, which is consistent with the predicted 58983 Da and 59280 Da molecular weight for Msmeq and Mtb respectively. Mtb and Msmeq MbtA share 69.2 % sequence identity and 85.0 % sequence similarity.

Cloning, Expression, and Purification of MbtB. The Mbt ACP domain of MbtB module was expressed in E. coli but was mostly insoluble. Using denaturing conditions, small quantities of soluble apo-MbtB-ACP were obtained after nickel affinity chromatography. As assessed by SDS-PAGE, apo-MbtB-ACP displayed an apparent molecular mass of ~11 kDa, consistent with the molecular weight of 11670 Da calculated from the amino acid sequence. For the subsequent activity assay with MbtA, apo-MbtB-ACP is required to be phosphopantetheinylated (holo-MbtB-ACP form). Sfp, a phosphopantetheinyl transferase from Bacillus subtilis (B. subtilis) was used to covalently transfer the 4'-phosphopantetheinyl group from coenzyme A onto apo-MbtB-ACP as described in the Materials and Methods section. The conversion of apo-MbtB-ACP to holo-MbtB-ACP was confirmed by addition of 340 Da moiety, corresponding to the phosphopantetheine arm by Fourier transform mass spectral analysis (data not shown).

Specific acetylation of MbtA by protein lysine acetyltransferase. Recently, protein lysine acetyltransferase (Pat) has been identified as a regulator of one of the later step of mycobactin biosynthesis via post-translational modification of fatty acyl-AMP ligase FadD33 (19). MbtA and FadD33 both catalyze an adenylation reaction followed by thioesterification of the substrate onto an ACP. FadD33 is acetylated by Pat on K511 and
amino acid sequence comparison of MbtA and FadD33 highlights a conserved lysine residue, K546, in MbtA (Fig. 2A). Moreover like FadD33-K511, MbtA-K546 is also flanked by a preceding glycine as well as two nearby downstream basic residues, which are believed to lower the steric hindrance between Pat and MbtA and reduce the pK\textsubscript{a} value of the NH\textsubscript{2} group of K546 respectively (Fig. 2A). Based on these observations we probed MbtA as a potential substrate for Pat. For this experiment Msme Pat (MSMEG_5458) was used instead of Mtb Pat (Rv0998) since protein refolding issues occurred after protein purification therefore affecting Mtb Pat activity. Western-blot analysis using anti-acetyllysine antibody shows that MbtA is acetylated by Pat in the presence of acetyl-CoA and cAMP (Fig. 2B). Based on these observations we probed MbtA as a potential substrate for Pat. For this experiment Msme Pat (MSMEG_5458) was used instead of Mtb Pat (Rv0998) since protein refolding issues occurred after protein purification therefore affecting Mtb Pat activity. Western-blot analysis using anti-acetyllysine antibody shows that MbtA is acetylated by Pat in the presence of acetyl-CoA and cAMP (Fig. 2B, lane 2). Negative controls without Pat or acetyl-CoA show no MbtA acetylation (Fig. 2B, lanes 3 and 4). Only in the negative control without cAMP, a light MbtA acetylation is observed (Fig. 2B, lane 5) likely due to co-purification of Pat with some bound cAMP as noted previously (23). Msme Pat also acetylates Mtb MbtA in the same manner (data not shown). To determine the site(s) of acetylation we created a single amino acid change in MbtA, changing K546 to an alanine. K546 appears to be the main acetylation site since the K546A mutant completely loses the ability to be acetylated by Pat by western-blot (Fig.2B, lane 1). To confirm this unique acetylation site, in vitro acetylated MbtA was analyzed by mass spectrometry. Good sequence coverage (85%) allowed the identification of K546 as the single acetylation site (Fig. 2C) and confirms that Pat acetylates MbtA on K546.

Effect of Acetylation on MbtA Activity. MbtA enzymatic activity was followed by monitoring the formation of AMP in the presence of 2,3-dihydroxybenzoate and acceptor holo-MbtB-ACP. To test acetylation effects, the activity of Msme MbtA was monitored overtime in the presence of Msme Pat, acetyl-CoA and cAMP. After 5 hrs of incubation, Msme MbtA activity was reduced by 80 % whereas MbtA activity without either acetyl-CoA or Pat remained unchanged (Fig. 3A). The gradual loss of activity is directly mediated by MbtA acetylation in a Pat- and acetylCoA-dependent manner. Using the same Mtb MbtA and Msme Pat ratio as used previously for Msme MbtA, 12 hrs of incubation were necessary to achieve complete Mtb MbtA inactivation (data not shown). For all subsequent experiments MbtAs were incubated overnight with Msme Pat to insure full inactivation. The Mtb genome encodes one known sirtuin-like deacetylase DAc (Rv1151c) in contrast to the Msme genome, which contains two sirtuin-like deacetylases named DAc1 (Msme_5175) and DAc2 (Msme_4620). DAc and DAc1 are very similar and were previously shown to deacetylate acetylated forms of acetyl-CoA synthetase (ACS) and FadD33 (19,23). Western-blot analysis of acetylated Mtb and Msme MbtA after overnight incubation with DAc deacetylase demonstrates that both MbtAs are deacetylated by DAc (Fig. 4A, lane 2 and 4B, lane 3). To check if deacetylation restores MbtA activity, acetylated MbtA was incubated with DAc and NAD\textsuperscript{+}. Over time, inactive acetylated MbtA regained its activity when incubated with NAD\textsuperscript{+} and DAc. However, omission of either NAD\textsuperscript{+} or deacetylase prevented reactivation (Fig. 3B). These results suggest that MbtA catalytic activity is regulated by Pat and DAc via the reversible acetylation and deacetylation of MbtA at position K546.

Crystal structure of Msme MbtA apo. Msme MbtA was crystallized in an apo form and the structure was solved to 2.3 Å (Table 1). The Msme MbtA structure was solved by molecular replacement using the DhbE structure (accession code: 1MDB), which is the adenylating domain in the bacillibactin siderophore biosynthesis (24). Molecular replacement led to a partial solution for MbtA with a defined N-terminal domain and two C-terminal domain fragments (residues 450-462 and 478-503). To complete the Msme MbtA structure, the C-terminal domain of DhbE was aligned with the MbtA C-terminal fragments. Through multiple cycles of refinements and manual corrections, a Msme MbtA model was generated containing one chain in the asymmetric unit with residues 18-151, 157-313, 321-556 observed and with a R\textsubscript{work} of 18.9 % and a R\textsubscript{free} of 24.5 %. The overall structure of Msme MbtA exhibits the typical fold observed for the ANL (Acyl/Aryl, NonRibosomal Peptide Synthetases and Luciferase) superfamily of adenylylating enzymes (25). The structure includes two main domains (Fig. 5A): a large N-terminal domain (1-457) and a smaller C-terminal domain (458-558). The N-terminal domain is composed of three
subdomains: two β-sheet subdomains (a and b) and a β-barrel subdomain (c). The subdomains (a) and (b) form a five-layered αβαβα sandwich. The subdomain (a) includes six β-strands and five α-helices while the second β-sheet subdomain contains eight β-strands and seven α-helices. The β-barrel subdomain (c) abuts (a) and (b) and leads to the compact C-terminal domain through a short hinge (including residue K457). The compact C-terminal domain consists of three α-helices and five β-strands and harbors K546, the lysine acetylated by Pat.

The Msmeg MbtA structure is very similar to the B. subtilis DhbE (44 % sequence identity) and the Acinetobacter baumannii BasE N-terminal domain (39 % sequence identity) structures. Superimposition of MbtA with DhbE and BasE N-terminal domain shows low r.m.s displacement values for the Ca atom positions of 1.0 Å and 0.7 Å respectively (Fig. 5B) (24,26). The highly conserved P-loop motif (S212-K221) is implicated in the ATP phosphate moiety binding and adopts a slightly different conformation compared to the BasE N-terminal domain or the DhbE structures due to the lack of substrate in the MbtA active site (24,26). Salmonella enterica acetyl-CoA synthetase (ACS) and MbtA both catalyzed adenylation reactions and structural superimposition indicates a fairly high structure similarity (4.9 Å r.m.s displacement for the Ca atom positions) (Fig. 5C) (27). Moreover, Pat acetylates ACS-K609 and MbtA-K546, which are both localized on the same C-terminal domain loop. The main difference between the Msmeg MbtA structure and the other structures (DhbE and ACS) lies in the conformation adopted by the C-terminal domain: the latter is rotated by 91° and 102° compared to the DhbE and the ACS C-terminal domains, respectively. The C-terminal domain forms a “lid”, which can close above the β-barrel subdomain and plays a significant role in the transfer of the adenylated product to the acyl-carrier protein (24,28). This domain has been shown to adopt different orientations depending on which half-reaction is catalyzed, either the adenylation or the thioesterification reactions (25,29).

Generation of precise null deletion mutants in genes encoding Pat (Rv0098) and DAc (Rv1151c) in Mtb. Prior to this study, the potential for regulation of mycobacterial siderophore production via post-translational acetylation of enzymes required for mycobactin biosynthesis has been demonstrated biochemically. Here, we also examine the in vivo impact of genetically removing Pat or Dac (Fig. 6A and 6C), under conditions encountered in the intraphagosomal environment. Towards this end, using a BLS2-safe M. tuberculosis H37Rv derivative, which is a double auxotroph for leucine and pantothenate, we engineered ∆Pat and ∆DAc Mtb mutant strains (Fig. 6B and 6D). The genes were deleted by the specialized transduction methodology described in the Materials and Methods. To verify the deletions, PCR amplification using three diagnostic independent primer pairs shows expected amplicon sizes for ∆Pat + pMV261 and ∆DAc + pMV261 (Fig. 6E and 6F).

∆Pat and ∆DAc share distinct phenotypes at low pH and limited iron conditions. Pat exhibits a unique structural feature with an N-terminal cAMP-binding domain fused to a C-terminal GNAT domain. Our current study and past studies (19,23) have shown that Pat acetylation activity is dependent on cAMP generated by multiple adenylate cyclases (AC). To investigate the effect of Pat and DAc deletions in ∆Pat and ∆DAc, we tested these strains under different physiological conditions such as low pH at which specific ACs induce cAMP production (22). As a reference point, we first determined growth curves for the five strains in Sauton medium at pH 7. We observed no significant growth difference between the Mtb WT + pMV261 (expression vector), Mtb ∆Pat + pMV261, Mtb ∆DAc + pMV261, Mtb ∆Pat + pMV261-Pat and Mtb ∆DAc + pMV261-DAc (Fig. 7A). All strains carried the pMV261 expression vector in order to facilitate a comparative analysis. In contrast, when these same strains were grown in Sauton medium at pH 6, Mtb ∆Pat + pMV261 showed a growth advantage compared to the parent and complemented strains, while Mtb ∆DAc + pMV261 displayed a slight growth defect (Fig. 7B). These results indicate that deletion of Pat leads to an acceleration of growth and suggests that lack of acetylation of Pat substrates at pH 6 is beneficial for Mtb. Since Pat is able to potentially inhibit mycobactin biosynthetic enzymes in vitro, we next cultured the five strains in Sauton medium with limiting iron (100 fold less than standard Sauton) to test the
same hypothesis that inhibition of siderophore production under these conditions would inhibit growth in vivo. In iron-limited Sauton medium at pH 7, we observed no significant difference in the growth of the strains, but a much slower growth rate was noted as compared with iron-sufficient media (Fig 7C). If we now compared the growth pattern in Sauton pH 6 with limited iron, we observed a distinct growth advantage for Mtb ΔPat + pMV261 compared to the parent and complemented stains, while Mtb ΔDAc + pMV261 displayed a major growth defect (Fig. 7D). These results support the idea that in vivo, at lower pH and under elevated cAMP levels, that deletion of Mtb Pat likely prevents the acetylation, and inhibition of MbtA and FadD33, thus allowing for mycobactin production, while deletion of Mtb DAc likely prevents the deacetylation of these enzymes and inhibits mycobactin production.

DISCUSSION

The acquisition of iron from the environment, its incorporation into iron-containing proteins and enzymes and the regulation of intracellular iron levels is essential for the growth and virulence of Mtb. While several methods for iron acquisition exist in the organism, the major mechanism is the production of the iron siderophore, mycobactin. This secondary metabolite has a number of unusual features. A hydroxypyphenoxazoline moiety, made from salicylate and serine, forms a N-cap to the adjacent εN-hydroxylysine residue that is additionally acylated on the ε-amino group. The remainder of the molecule contains a hydroxybutyryl group and a terminal εN-hydroxylysine residue that is cyclized into a seven-membered hydroxamate ring. The hexadentate coordination of ferric iron occurs from the six oxygen and nitrogen atoms in the molecule shown in blue in Figure 1A. Acylation of the central hydroxylysine residue occurs with saturated and Δ2-unsaturated long chain fatty acids to generate mycobactin or with short chain dicarboxylic acids to generate carboxymycobactins. It is thought that the carboxymycobactins are secreted and acquire host iron and then deliver this iron to the membrane-associated mycobactins (10).

It has been recognized for some time that bacteria tightly regulate the production of siderophores such as mycobactin. In Mtb, a major regulator of mycobactin production is the iron-binding transcriptional regulator IdeR (Rv2711). This 230-residue protein is essential for mycobacterial growth and is a member of the diphtheria toxin repressor family and exists as a homodimer in solution (30). It binds to a highly conserved 19 base pair inverted repeat that is found adjacent to genes whose transcription is repressed at high iron concentrations. In both the Mbt-1 and Mbt-2 gene clusters, multiple IdeR binding sites are observed adjacent to the transcriptional start sites for the genes in the two clusters. As iron is acquired by the mycobactins and the intracellular concentration increases, the iron-bound IdeR binds to these regions, effectively preventing transcription of these genes (31). This is a general mechanism of regulation in many bacteria. There is a recent report that the HupB protein can serve as a positive regulator of mycobactin synthesis under conditions of low intracellular iron by binding to a region 5’ to that of the IdeR binding site and recruiting RNA polymerase to the transcription start site (32).

In the current study, we have analyzed the potential for non-transcriptional regulation of mycobactin biosynthesis. We have previously reported the reversible, post-translational monoacetylation of both the Mtb acetyl-CoA synthetase (ACS) and the mbtM-encoded acyl-ACP synthetase (FadD33) (19,23). These enzymes are mechanistically related and use ATP to form an intermediate acetyl or fatty acyl adenylate, which is then nucleophilically attacked by the thiol of CoASH or the phosphopantetheine arm of an ACP domain. Knowing the primary sequence around the lysine residue that is acetylated in both these enzymes, we identified a highly similar sequence in the Mtb and Msmeg MbtA enzymes. It was previously shown that MbtA-K546 lysine equivalents in FadD33 and ACS are required for catalysis in the first adenylation half reaction (19,23). The mechanism of MbtA is similar to that of both ACS and FadD33 in that a carboxyl group of the substrate is initially adenylated for activation, followed by the attack of a thiol group on the mixed carboxylic-phosphoric anhydride, although MbtA uniquely uses an aryl carboxylate, salicylate. We now show that MbtA is also reversibly acetylated and that this leads to enzyme activity loss.
MbtA has become a well-established druggable target in the last decade. The production of mycobactin is essential and the tight binding of hydrolytically stable, isosteric analogs of the salicyl adenylate has been demonstrated (33). The intermediate analog, 5’-O[N-(salicyl)sulfamoyl]adenosine was shown independently by three groups to inhibit MbtA with a Ki of ~ 6 nM (33-35). This compound in an acute mouse model. Since MbtA is the first and committed step in mycobactin synthesis, it is perhaps not surprising that its inhibition would be so deleterious.

To support our biochemical analysis we also performed genetic analysis to investigate the role of acetylation in the Mtb physiological contest. We generated two strains in which either the Pat or DAc genes were knocked-out, and strains in which the knockouts were complemented. To mimic the environment inside the macrophage phagosome, strains were grown at low pH and limiting iron condition. While at pH 7 (Sauton pH7 and limited iron pH7), all strains exhibit a similar growth phenotype. But at relevant physiological pH (pH 6 and under limiting iron conditions), the ΔPat strain grows significantly faster than the WT, and the ΔDAc grows considerably slower. Both complemented strains grow approximately as well as WT. This suggests that when acetylation of MbtA or FadD33 is prevented in the ΔPat strain, the resulting active enzymes are capable to generating the required levels of mycobactin. On the other hand, in the ΔDAc strain, MbtA and FadD33 are likely to be functioning at less than full activity, even less activity than the WT strain resulting in insufficient mycobactin levels. Thus mutant phenotypes suggest that acetylation may influence Mtb survival in the macrophage.

It is clear that the transcriptional regulation of the mycobactin biosynthetic gene clusters is the major mechanism by which mycobactin biosynthesis is regulated. However, once the proteins are made, they remain constitutively active, potentially enabling for the production of excess mycobactin, and allowing for the accumulation of intracellular iron in excess of its ability to be incorporated into iron-dependent proteins. This would lead to free iron in the cell, and in the reducing, but aerobic, intracellular environment of the cell, the potential for the generation of reactive oxygen species through the action of Fenton-type chemistry. In support of this, a recent report showed that Mtb is particularly sensitive to ascorbic acid, Vitamin C, in comparison to other Gram negative and positive species (36). The ability to rapidly inactivate MbtA could be used to break the ATP-consuming production of mycobactin very quickly when the organism senses that sufficient iron is present. This secondary level of regulation could be used to fine tune iron levels, allowing just enough as required and preventing the over accumulation of iron to toxic levels.

EXPERIMENTAL PROCEDURES
Cloning, Expression, and Purification of MbtA. MbtA (MSMEG_4516 and Rv2384) was amplified from Msmeg mc²155 and Mtb H37Rv genomic DNA using the primer pairs Msme MbtA_F (5’-GGAATTCCATATGACTCTGACCAAGCCTCCACACGC-3’) and Msmeg_MbtA_R (5’-CCCCAGGTTCCCGCGAGGCTAGCCACGGCAGTGA-3’) or Mtb_MbtA_F (5’-GGAATTCCATATGGCCACCGAGGCGCGAGCTAGCCACGGCAGTGA-3’) and Mtb_MbtA_R (5’-CCCCAGGTTCCCGCGAGGCTAGCCACGGCAGTGA-3’) containing Ndel and HindIII sites, respectively. The PCR amplicon was ligated into the pET-28a (+) vector, and then transformed into E. coli DH5α competent cells to create pET-28a (+)::MbtA N-terminally His₆ tag plasmid. A sequence verified construct was transformed in E. coli T7 express lys’/P for protein expression. A 4 mL preculture was used to inoculate 1 L of Luria-Bertani medium supplemented with 50 µg/mL kanamycin. The culture was grown to midlog phase (A₆₀₀ ~ 0.6) at 37 °C, then induced by the addition of 0.4 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG). After 18 hrs of additional incubation (20 °C), cells were harvested by centrifugation (6,000 g, 20 min) and stored at -80 °C. The cell pellet was thawed and resuspended in lysis buffer (25 mM Tris, pH 8.0 containing 150 mM NaCl, 10 mM imidazole) supplemented with DNasel (0.1 µg/mL), lysozyme (2 mg/mL) and EDTA-free protease inhibitors cocktail (Roche).
Resuspended cells were disrupted by sonication and cellular debris was removed from the lysate by centrifugation at 16000 rpm for 50 min. MbtA was purified by nickel affinity chromatography. After an extensive wash with 25 mM Tris, pH 8.0 containing 150 mM NaCl and 10 mM imidazole buffer, the bound protein was eluted with a linear imidazole gradient (30-250 mM). Pure fractions, as determined by SDS-PAGE, were pooled together for buffer-exchange into 25 mM Tris, pH 8.0 containing 150 mM sodium chloride and 10 % glycerol. For crystallization purpose, the N-terminal polyhistidine tag of Msmeg MbtA was cleaved using thrombin protease. The cleavage reaction was dialyzed overnight against 50 mM Tris (pH 8.0), 150 mM sodium chloride, 1 mM dithiothreitol and 1 mM calcium chloride. The enzyme was further purified using size exclusion chromatography (HiLoad™ 26/60 Superdex™ 75 prep grade) in the buffer described above without calcium chloride and then concentrated to 12 mg/mL using a 30-kDa Amicon Ultra Centrifugal filter.

Crystallization. The apo enzyme was screened against the MCSG suite (Microlytic, 384 conditions). A Crystal Gryphon robot (Art Robbins) was used to dispense both the reservoir solutions and the sitting-drops (1:1 ratio). Crystal trays (Intelli-Plates 96, Art Robbins) were then sealed and incubated at 20 °C. Preliminary crystals were obtained in the condition 1D2 containing 0.2 M sodium chloride, 0.1 M bis-tris (pH 6.5) and 25 % (w/v) polyethylene glycol 3350. The crystallization condition containing 0.1 M bis-tris (pH 6.5), 0.4 M sodium chloride and 17.5 % (w/v) polyethylene glycol 3350 improved the quality of the crystal. The latter was cryoprotected by addition of 30 % (w/v) polyethylene glycol and flash-cooled in liquid nitrogen.

Data collection and structure determination. Diffraction data were collected at the Lilly Research Laboratories Collaborative Access Team (LRL-CAT) beamline at the Advance Photon Source Argonne National Laboratory (APS-ANL, IL), at 0.97931 Å wavelength radiation. Diffraction was observed to 2.3 Å. Data was collected at 2.3 Å. Data was indexed, integrated and finally scaled using the XDS package (37). Matthews’ coefficient analysis indicated the presence of one molecule in the asymmetric unit (43 % solvent). The structure of DhbE from *B. subtilis* (PDB entry 1MDF) was used to carry out molecular replacement with PHASER-MR (24,38). The solution obtained by molecular replacement was used as a template for the Autobuild tool to build 78 % of the model (39). The rest of the model was manually built using COOT and iterative cycles of refinements (rigid body refinement, simulated annealing, positional and B-factor refinements) (40,41). Three residues were observed in the disallowed region of the Ramachandran plot (V197, V544, R554).

Cloning, Expression, and Purification of MbtB-ACP. The acyl carrier protein domain of Mbt enzyme, MbtB-ACP (Rv2383c) was amplified from *Mtb* genomic DNA using the primer pairs *Mtb MbtB-ACP_F* (5’-GGAGGGCATATGGTGATGCTACGGCG-3’) and *Mtb MbtB-ACP_R* (5’-CCCAGCTTTGCGCACTGCGTGGG-3’), containing NdeI and HindIII sites, respectively. The PCR amplicon was ligated into the pET-28a (+) vector, and then transformed into *E. coli* DH5α competent cells to create pET-28a (+)::MbtB-ACP N-terminally Hisα tag plasmid. A sequence verified construct was transformed in *E. coli* T7 express lys/f’ for protein expression. MbtB-ACP domain was expressed as per MbtA and cells were stored at -80 °C. The cell pellet was thawed and resuspended in lysis buffer (25 mM Tris, pH 8.0) supplemented with DNaseI (0.1 µg/mL) and EDTA-free protease inhibitors cocktail (Roche). Resuspended cells were disrupted by homogenization using the EmulsiFlex-C3 (Avestin) at 10000 psi and cellular debris was removed from the lysate by centrifugation at 16000 rpm for 50 min. MbtB-ACP was purified by nickel affinity chromatography. After extensive wash with 4 M urea, 25 mM Tris, pH 8.0, 10 mM imidazole buffer, the bound protein was eluted with a linear imidazole gradient (30-250 mM) with 4M urea. Pure fractions, as determined by SDS-PAGE were pooled together for buffer-exchange first in 1 M urea, 50 mM Tris, pH 8.0, 150 mM NaCl and then in 50 mM Tris, pH 8.0, 150 mM NaCl to allow refolding. MbtB-ACP was concentrated using an Amicon pressure concentrator with a 3 K Dalton filter to 100 µM.

Cloning, Expression, and Purification of Sfp. Sfp was amplified from *B. subtilis* genomic DNA using the primer pairs *Bsub Sfp_F* (5’-GGTATTGGAGGTTCGATGAAGATTTACGG-3’) and *Bsub Sfp_R* (5’-GGTATTGGAGGTTCGATGAAGATTTACGG-3’). The PCR amplicon was ligated into the pET-28a (+) vector, and then transformed into *E. coli* DH5α competent cells to create pET-28a (+)::Sfp plasmid. A sequence verified construct was transformed in *E. coli* T7 express lys/f’ for protein expression. Sfp domain was expressed as per MbtA and cells were stored at -80 °C. The cell pellet was thawed and resuspended in lysis buffer (25 mM Tris, pH 8.0) supplemented with DNaseI (0.1 µg/mL) and EDTA-free protease inhibitors cocktail (Roche). Resuspended cells were disrupted by homogenization using the EmulsiFlex-C3 (Avestin) at 10000 psi and cellular debris was removed from the lysate by centrifugation at 16000 rpm for 50 min. Sfp was purified by nickel affinity chromatography. After extensive wash with 4 M urea, 25 mM Tris, pH 8.0, 10 mM imidazole buffer, the bound protein was eluted with a linear imidazole gradient (30-250 mM) with 4M urea. Pure fractions, as determined by SDS-PAGE were pooled together for buffer-exchange first in 1 M urea, 50 mM Tris, pH 8.0, 150 mM NaCl and then in 50 mM Tris, pH 8.0, 150 mM NaCl to allow refolding. Sfp was concentrated using an Amicon pressure concentrator with a 3 K Dalton filter to 100 µM.

Cloning, Expression, and Purification of MbT-ACP. The acyl carrier protein domain of MbT enzyme, MbT-ACP (Rv2383c) was amplified from *Mtb* genomic DNA using the primer pairs *Mtb MbT-ACP_F* (5’-GGAGGGCATATGGTGATGCTACGGCG-3’) and *Mtb MbT-ACP_R* (5’-CCCAGCTTTGCGCACTGCGTGGG-3’), containing NdeI and HindIII sites, respectively. The PCR amplicon was ligated into the pET-28a (+) vector, and then transformed into *E. coli* DH5α competent cells to create pET-28a (+)::MbT-ACP N-terminally Hisα tag plasmid. A sequence verified construct was transformed in *E. coli* T7 express lys/f’ for protein expression. MbT-ACP domain was expressed as per MbtA and cells were stored at -80 °C. The cell pellet was thawed and resuspended in lysis buffer (25 mM Tris, pH 8.0) supplemented with DNaseI (0.1 µg/mL) and EDTA-free protease inhibitors cocktail (Roche). Resuspended cells were disrupted by homogenization using the EmulsiFlex-C3 (Avestin) at 10000 psi and cellular debris was removed from the lysate by centrifugation at 16000 rpm for 50 min. MbT-ACP was purified by nickel affinity chromatography. After extensive wash with 4 M urea, 25 mM Tris, pH 8.0, 10 mM imidazole buffer, the bound protein was eluted with a linear imidazole gradient (30-250 mM) with 4M urea. Pure fractions, as determined by SDS-PAGE were pooled together for buffer-exchange first in 1 M urea, 50 mM Tris, pH 8.0, 150 mM NaCl and then in 50 mM Tris, pH 8.0, 150 mM NaCl to allow refolding. MbT-ACP was concentrated using an Amicon pressure concentrator with a 3 K Dalton filter to 100 µM.

Cloning, Expression, and Purification of MbT-ACP. The acyl carrier protein domain of MbT enzyme, MbT-ACP (Rv2383c) was amplified from *Mtb* genomic DNA using the primer pairs *Mtb MbT-ACP_F* (5’-GGAGGGCATATGGTGATGCTACGGCG-3’) and *Mtb MbT-ACP_R* (5’-CCCAGCTTTGCGCACTGCGTGGG-3’), containing NdeI and HindIII sites, respectively. The PCR amplicon was ligated into the pET-28a (+) vector, and then transformed into *E. coli* DH5α competent cells to create pET-28a (+)::MbT-ACP N-terminally Hisα tag plasmid. A sequence verified construct was transformed in *E. coli* T7 express lys/f’ for protein expression. MbT-ACP domain was expressed as per MbtA and cells were stored at -80 °C. The cell pellet was thawed and resuspended in lysis buffer (25 mM Tris, pH 8.0) supplemented with DNaseI (0.1 µg/mL) and EDTA-free protease inhibitors cocktail (Roche). Resuspended cells were disrupted by homogenization using the EmulsiFlex-C3 (Avestin) at 10000 psi and cellular debris was removed from the lysate by centrifugation at 16000 rpm for 50 min. MbT-ACP was purified by nickel affinity chromatography. After extensive wash with 4 M urea, 25 mM Tris, pH 8.0, 10 mM imidazole buffer, the bound protein was eluted with a linear imidazole gradient (30-250 mM) with 4M urea. Pure fractions, as determined by SDS-PAGE were pooled together for buffer-exchange first in 1 M urea, 50 mM Tris, pH 8.0, 150 mM NaCl and then in 50 mM Tris, pH 8.0, 150 mM NaCl to allow refolding. MbT-ACP was concentrated using an Amicon pressure concentrator with a 3 K Dalton filter to 100 µM.
AATT-3') and  Bsub_Sfp_R (5'-AGAGGGAGTTAGAGCCTTATAAAGGCCTTCGGC TTCGTA-3'). The PCR amplicon was ligated into the pET-30 Xa/LIC (+) vector, and then transformed into E. coli DH5α competent cells to create pET-30 Xa (+)::Sfp N-terminally His6 tag plasmid. A sequence verified construct was supplemented with 50 µg/mL kanamycin. The culture was grown to midlog phase (A600 ~ 0.6) at 37 ºC, then induced by the addition of 0.1 mM IPTG. After 18 hrs of additional incubation (30 ºC), cells were harvested by centrifugation (6,000 g, 20 min) and stored at -80 ºC. The cell pellet was thawed and resuspended in lysis buffer (50 mM sodium phosphate, pH 8.0 containing 300 mM NaCl, 10 mM imidazole) supplemented with DNaseI (0.1 µg/mL), lysozyme (2 mg/mL) and EDTA-free protease inhibitors cocktail (Roche). Resuspended cells were disrupted by sonication and cellular debris was removed from the lysate by centrifugation at 16000 rpm for 50 min. Sfp was purified by nickel affinity chromatography. After an extensive wash with 50 mM sodium phosphate, pH 8.0 containing 300 mM NaCl and 10 mM imidazole buffer, the bound protein was eluted with a linear imidazole gradient (30-250 mM). Pure fractions, as determined by SDS-PAGE, were pooled together for buffer-exchange into 50 mM sodium phosphate, pH 8.0 containing 150 mM NaCl, 5 mM DTT, 10 mM MgCl2 and 10 % glycerol. Sfp was concentrated using an Amicon pressure concentrator with a 10 K Dalton filter to 250 µM.

**Phosphopantetheinylation of MbtB-ACP by Sfp.** To convert the apo form of MbtB-ACP to the phosphopantetheinylated form, holo-MbtB-ACP, the phosphopantetheinyl transferase Sfp was used. 100 µM Apo-MbtB-ACP was incubated with 0.2 µM Sfp in 50 mM Tris, pH 7.8 with 150 mM NaCl, 1 mM CoASH and 2 mM DTT. The reaction was allowed to proceed at 25 ºC for 18 hrs before buffer-exchange into 50 mM Tris, pH 8.0, 150 mM NaCl. Traces of Sfp were removed during MbtB-ACP concentration using two successive Amicon filtrations, 30 K Dalton and 3 K Dalton cut-off filters respectively.

**Measurement of Enzymatic Activity.** The enzymatic activity of MbtA was determined spectrophotometrically by coupling the formation of AMP to the reactions of myokinase, pyruvate kinase and lactate dehydrogenase as described previously (42). Reactions were performed in 100 mM Hepes pH 7.5, 10 mM MgCl2, 250 mM NaCl, 1 mM PEP, 0.15 mM NADH, 300 µM holo-MbtB-ACP, 18 units of myokinase, 18 units of pyruvate kinase, and 18 units of lactate dehydrogenase in a final volume of 100 µL. Typically 0.5 µM of Msme or Mbt MbtA was used and incubated for 5 min at 25 ºC with the reaction mix prior to reaction initiation by substrate addition with 2,3-dihydroxybenzoate, a salicylic acid analogue. The reaction was monitored at 340 nm (ε340 = 6220 M⁻¹ cm⁻¹) using a Shimadzu spectrophotometer (UV-2450).

**Site-Directed Mutagenesis and Purification of MbtA-K546A.** The K546A mutation was introduced into the pET-28a (+)::MbtA N-terminally His6 tag plasmid using the Quickchange mutagenesis kit (Stratagene) with the following primers  Mbt_MbtA_K542A_F (5'-CAACGCCGATCGGGCGATCGACAAACGAG-3'),  Mbt_MbtA_K542A_R (5'-CTCGTTTGTGATCGCCCGATCGGCTGTG-3'),  Msme_MbtA_K546A_F (5'-CACGGCCGTCGGCGATCGAACAAGAGAGGAGAG-3') and  Msme_MbtA_K546A_R (5'-CTCTTTGTGATCGCCCGATCGGCTGTG-3'). The mutation was confirmed by DNA sequencing. The expression and purification of MbtA mutant was the same as described for the wild type.

**In vitro Acetylation Assay.** 10 µM MbtA or the mutant protein were incubated with 1 mM cAMP, 100 µM AcCoA and 1 µM Msme Pat at 37 ºC for 5 hours. Samples were then analyzed by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. Western blots were performed using an anti-acetyllysine antibody (Cell Signaling Technology Inc. and ImmuneChem Pharmaceuticals Inc., dilution of 1:2000) and Goat Anti-Rabbit IgG AP conjugate (Bio-Rad Laboratories, dilution of 1:2000). Development was carried out according to the manufacturer’s instructions.

**Time-Dependent Inactivation and reactivation of MbtA.** 20 µM MbtA was incubated with 1 mM cAMP and 100 µM AcCoA with 5 µM Msme Pat in 50 mM HEPES pH 7.5, 100 mM NaCl at 37 ºC. Aliquots of the reaction mixture were withdrawn
when required, kanamycin (25 µg/ml) was added to the medium. Sauton, 7H9 and Sauton iron-limited medium pHs were adjusted to 7.0 or 6.0 and 100 mM MES was added to maintain a constant pH during growth curve.

Rv0998 and Rv1151c mutant constructions in Mtb. The gene knockouts of Pat acetyltransferase (Rv0998) and Sirtuin-like deacetylase DAc (Rv1151c) were created by specialized transduction methodology in mc²6206 (44,45). Constructs for allelic exchange were generated by amplifying the upstream and downstream flanking regions of each of the two genes using the primer pairs listed in Table 2. The upstream and downstream flanking regions were cloned into suicidal delivery vector pYUB1471 to create an allelic exchange vectors harboring a selectable/counterselectable cassette [γδ(sacB-Hyg)γδ] between the two flanking regions. The allelic exchange constructs were incorporated into shuttle mycobacteriophage vector phAE159; the phasmid constructs used in this work were obtained as part of a collaboration with Genomics Institute of the Novartis Research Foundation (GNF) to generate a set of gene deletion constructs for Mtb (46). Phasmid DNA was electroporated into Msme to obtain plaques at the permissive temperature of 30 °C. Specialized transducing phages were picked and amplified at 30 °C to generate high titer mycobacteriophage. mc²6206 was transduced with high-titer phages at the non-permissive temperature of 37 °C to delete genes of interest by specialized transduction (44,45). The transductants were plated on selective medium: Middlebrook 7H10 medium (Difco) containing 10% OADC enrichment (0.5 g oleic acid, 50 g albumin, 20 g dextrose, 0.04 g catalase, 8.5 g sodium chloride in 1 L water), 0.5% glycerol, 50 µg/ml leucine, 24 µg/ml pantothenate and 50 µg/ml hygromycin. Genomic DNA prepared from transductants was screened by a three-primer PCR to confirm gene deletion, using primers in Table 3.

For complementation, the Rv0998 and Rv1151c genes were amplified by PCR from H37Rv genomic DNA using the following primers Rv0998_F (TTTTTTTTGGATCCATTGGACGGATAGCGAATTC), Rv0998_R (TTTTTTTTGTTACTCAGCCGAGCTCGATCAC), Rv1151c_F (TTTTTTTTGGATTTGATGCAGTTGGCGGT...
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GCTCAG), and Rv1151c_R (TTTTTTTTGTTAACCTATTTTCAGCAGGCGGCAG) containing BamHI and HpaI respectively. PCR products were digested with BamHI and HpaI and cloned into the mycobacterial episomal pMV261 vector using the BamHI and HpaI restriction sites (47). After sequence verification, complementation plasmids were electroporated into the relevant deletion mutant strains, with transformants selected on 7H10 medium as noted above, also containing 25 mg/ml kanamycin. To generate control strains for comparison in growth studies, the pMV261 empty vector was similarly electroporated into mc26206 parental, mc26206ΔPat and mc26206ΔDAc.

Growth assays. Mycobacterial strains were inoculated in liquid media, 7H9 pH 7 and then transferred into fresh 7H9, Sauton or low-iron Sauton media at either pH 7 or pH 6. After one culture passage in the final medium to allow strain adaptation, growth curves were started out at an OD600 = 0.05 and optical densities were measured at regular intervals for 20-40 days.
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AUTHOR CONTRIBUTIONS
OV, HX, JMT, JSB and WRJ planned and designed research. OV purified and LF crystallized MbtA protein and determined its X-ray structure. OV and HX performed the in vitro enzyme activity. JMT designed mutant constructs and mutants were obtained by HX and OV. OV, HX and AAM analyzed the mutant phenotypes. OV, LF and JSB wrote the manuscript. All authors reviewed the results and approved the final version of the manuscript.

The authors declare that they have no conflicts of interest with the contents of this article

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Salicyl-capped mycobactin siderophore structure, genetic loci and first biosynthesis reaction. A, lipophilic mycobactin (MBT) and hydrophilic carboxymycobactin (cMBT) share a common core structure but differ in the length of the alkyl substitution (R group). Atoms in blue are involved in the hexadentate ferric iron coordination. B, mbt-1 gene cluster produces mycobactin core whereas mbt-2 gene cluster assembles and loads acyl fatty acid onto mycobactin lysine core. The black boxes indicate the presence of IdeR binding sequences, which cause genes repression upon iron binding on IdeR. C, adenylation-ligation reaction catalyzed by MbtA. ACP: acyl carrier protein domain of MbtB.

Figure 2. Pat acetylates specifically MbtA on Lys-546. A, Partial alignments of MbtA proteins from M. smegmatis (MSMEG_4516) and M. tuberculosis (Rv2384) versus M. smegmatis FadD33 (MSMEG_2132). Acetylated lysines are indicated in bold. Conserved residues and basic residues flanking acetylated sites are underlined. B, wild type MbtA or MbtA K546A mutant was incubated with multiple reaction components as indicated in the table. The samples were analyzed by western-blott (bottom panel) with acetyl-lysine antibody (α-AcK) and total protein content was determined by Ponceau red (top panel). C, MbtA unique acetylation site was identified by MS/MS as Lys-546. Shown in an MS/MS spectrum charged tryptic peptide from Ms MbtA (TTAVGKAcIDKK) bearing an acetylated lysine. The acetylated lysine is indicated as KAc.

Figure 3. Acetylation inhibits and deacetylation enhances the MbtA enzyme activity. A, Time-dependent inactivation of Msmeg MbtA by acetylation. Msmeg MbtA activity was monitored at different time intervals with the additional components: (●): cAMP, Msmeg Pat and acetyl-CoA; (▲): cAMP and acetyl-CoA; (△): cAMP and Msmeg Pat. B, Time-dependent reactivation of acetylated Mtb MbtA by deacetylation. After acetylation by Msmeg Pat, Mtb MbtA was then incubated with the following components: (●): NAD+ and Mtb DAc; (▲): Mtb DAc; (○): NAD+.

Figure 4. Mtb DAc (Rv1151c) enzyme deacetylates both MbtA homologues. A, Acetylated Mtb MbtA were analyzed by western-blott (bottom panel) with acetyl-lysine antibody (α-AcK) and total protein content was determined by Ponceau red (top panel). B, Acetylated Msmeg MbtA were analyzed by western-blott (bottom panel) with acetyl-lysine antibody (α-AcK) and total protein content was determined by Ponceau red (top panel).

Figure 5. Crystal structure of apo Msmeg MbtA. A, Stereo view of the overall structure of apo Msmeg MbtA. The three subdomains parts of the N-terminal domain are colored in purple (a), blue (b) and cyan (c), respectively, while the C-terminal domain, or “lid”, is represented in orange. The location of the active site is highlighted by a star. The residue K546, demonstrated in this manuscript to be acetylated, is rendered as sticks colored by CPK, with carbon atoms in orange. B, Superimposition of the apo Msmeg MbtA (green), B. subtilis DhbE in complex with DHB adenyalted (blue, pdb accession code: 1MDB) and Acinetobacter baumannii BasE N-terminal domain (yellow, pdb accession code: 3O83) structures. The...
DHB-adenylate product is displayed as sticks colored by CPK, with carbon atoms in magenta. C, Superimposition of the apo *Msmeg* MbtA (green) and *Salmonella enterica* acetyl-CoA synthetase in complex with CoA and AMP (orange, pdb accession code 2P2F) structures. The CoA and AMP ligands are rendered as sticks colored by CPK, with carbon atoms in white.

**Figure 6. Construction and verification of Mtb *Pat* (*Rv0998*) and Mtb *DAc* (*Rv1151c*) deletion mutants.** A and C, *Rv0998* and *Rv1151c* adjacent gene organisations are shown. B and C, the ORF removal in Δ*Rv0998* and Δ*Rv1151c* deletion strains are replaced by sacB-hyg^R^ cassette. E and F, left and right flanking PCR were used to confirm deletion of *Rv0998* and *Rv1151c* with specific set of primers.

**Figure 7. Iron level and pH effects on Mtb *Pat* and Mtb *DAc* deletion mutant phenotypes.** Four growth conditions are presented: A, Sauton pH7 medium; B, Sauton pH6 medium; C, limited iron Sauton pH7 medium and D, limited iron Sauton pH6 medium for five constructs. (black), *Mtb* wild type + pMV261; (solid red), *Mtb* Δ*Pat* + pMV261; (dotted red), *Mtb* Δ*DAc* + pMV261; (solid green), *Mtb* Δ*Pat* + pMV261-Pat and (dotted green), *Mtb* Δ*DAc* + pMV261-DAc. Error bars represent standard deviations from the mean of results from biological duplicates. *Mtb* wild type corresponds to mc^2^6206.
### TABLES

#### Table 1. Data collection and refinement statistics.

| Data Collection | MsMbtA apo |
|-----------------|------------|
| **PDB ID**      | 5KEI       |
| **Space Group** | P2₁₂₁₂     |
| **Unit Cell Dimensions** |        |
| \(a, b, c\) (Å) | 56.4; 85.8; 104.7 |
| \(\alpha, \beta, \gamma\) (°) | 90.0; 90.0; 90.0 |
| **Resolution Range** (Å) | 50.0-2.3 |
| **Wavelength** (Å) | 0.97931 |
| **R_{merge} (%)** (Highest shell) | 12.8 (79.3) |
| **CC(1/2) (Highest shell)** | 99.3 (65.5) |
| **I/\sigma(I)** (Highest shell) | 8.7 (1.6) |
| **Completeness (%)** (Highest shell) | 99.6 (98.8) |
| **Multiplicity** (Highest shell) | 3.9 (3.8) |
| **Total Reflections** (Unique) | 163230 (41974) |
| **Refinement statistics** |  |
| \(R_{work}/R_{free}\) | 18.9/24.5 |
| **Number of non-hydrogen atoms** |  |
| Protein | 3943 |
| Water | 102 |
| **Average B-factors (Å²)** | 40.3 |
| Protein | 40.3 |
| Water | 35.6 |
| **Wilson B factor (Å²)** | 35.8 |
| **R.m.s. deviations** |  |
| Bond lengths (Å) | 0.008 |
| Bond angles (°) | 1.169 |
| **Ramachandran plot** |  |
| Favor(P%) | 95.0 |
| Outliers(%) | 0.6 |
### Table 2. PCR primers for cloning flanks of the indicated loci, to generate allelic exchange substrates.

| Locus | Flank | Primer name | Primer sequence |
|-------|-------|-------------|-----------------|
| Rv1151c | Left | DRv1151c LL | TTTTTTTTCCATAAATTGGCCGGACCTGGTAAAATAA |
|        | Left | DRv1151c LR | TTTTTTTTCCATTTCTTGGTACAAAGAATTGCGCTTG-GATGTCTCAGTGAGGTCTTCTTCTTGCTATCGGCCGGACG |
|        | Right | DRv1151c RR | TTTTTTTTCCATCTTTTGCCAAGATCCATGCCCTGACC |
|        | Right | DRv1151c RL | TTTTTTTTCCATATGATTGGCTGGTCTAGTAGTGTATAGC-GCAGTGTCAGTGTTAGGTATCCGAGATCGATCGATCCATCCGCGAGT |
| Rv0998 | Left | DRv0998 LL | TTTTTTTTCCATAAATTGGACGAAGGCATTCCGGCAAA |
|        | Left | DRv0998 LR | TTTTTTTTCCATTTCTTGGAATCGTATGACACGCGCTTGG-ATGTCTCAGTGAGGTCTTCTCCGACATCCCTGAAAGACG |
|        | Right | DRv0998 RR | TTTTTTTTCCATCTTTTGAGCTCGCTCCGGGAAAAAGT |
|        | Right | DRv0998 RL | TTTTTTTTCCATAGATTGGTAATGATTGGCTGAGC-AGTGTCAGTGCTTCTCGAGTCTGAGCTGAGGTTG |

### Table 3. PCR Primers used to screen for deletions at the indicated loci.

| Locus | Flank | Primer name | Binding site | Primer sequence |
|-------|-------|-------------|--------------|-----------------|
| Rv1151c | Left | 1151c L Flank F=P7 | Upstream of L flank, in wild-type and mutant | CAT GCC GTC CAG CAT GTC |
|        |       | sacBout_LR=P10 | Within AES construct, mutant only | GAT GTC TCA CTG AGG TCT CT |
|        | Right | 1151c R Flank F=P5 | Within deleted region, wild-type only | CGT CAT CAC CCA GAA TGT C |
|        |       | hygout_RR= P9 | Within AES construct, mutant only | CGA GTG TCT GGT CTC GTA G |
|        |       | 1151c R Flank R=P6 | Downstream of R flank, in wild-type and mutant | GGC ACT GTC GGA TTA CAA G |
| Rv0998 | Left | 0998 L Flank F=P1 | Upstream of L flank, in wild-type and mutant | CGT TGT GTC TAC TGC TCG AC |
|        |       | sacBout_LR=P9 | Within AES construct, mutant only | GAT GTC TCA CTG AGG TCT CT |
|        |       | 0998 L Flank R=P2 | Within deleted region, wild-type only | GAT GAT CGC AAC ACC ATC |
|        | Right | 0998 R Flank F=P3 | Within deleted region, wild-type only | CGG TTC ATG TCG GCT CGT GGT C |
|        |       | hygout_RR= P10 | Within AES construct, mutant only | CGA GTG TCT GGT CTC GTA G |
|        |       | 0998 R Flank R=P4 | Downstream of R flank, in wild-type and mutant | TGT GCG GTA CAT CGA CCA CCT C |
FIGURES

Figure 1.
Figure 2.

A  

| Protein          | Peptide Sequence | MW  |
|------------------|------------------|-----|
| Msmeg MbtA       | SLPTAVGK       | 538 |
| Mtb MbtA         | ALPTTPIGKIDKRA | 534 |
| Msmeg FadD33     | SLPRTSGLRRLE   | 503 |

B  

- Ponceau red: 75 kDa, 50 kDa
- α-AcK: 75 kDa, 50 kDa

| Condition | MbtA | MbtA<sub>K546A</sub> | MsPat | AcCoA | cAMP |
|-----------|------|-----------------------|-------|-------|------|
| -         | +    | -                     | +     | +     | +    |
| +         | -    | -                     | +     | -     | +    |
| +         | +    | -                     | +     | -     | +    |

C  

Legend:   
- G   | V  | A  | H  | S  | P  | D  | A  | L  | V  | P  | S  | L  | P  | T  | A  | V  | G<sup>bc</sup> | I  | D  | K  | K  

Mass Spectrum:  
- Relative Abundance: 0 to 100
- m/z: 400 to 1724.74
Figure 4.

A

|   | 1  | 2  | 3  | MW |
|---|----|----|----|----|
| Ponceau red |    |    |    | 75kDa |
| α-AcK       |    |    |    | 50kDa |
| Mtb MbtA-Ac | +  | +  | +  | 75kDa |
| NAD⁺        | +  | +  | -  | 50kDa |
| Mtb DAc     | -  | +  | +  |     |

B

|   | 1  | 2  | 3  | MW |
|---|----|----|----|----|
| Ponceau red |    |    |    | 75kDa |
| α-AcK       |    |    |    | 50kDa |
| Msme MbtA-Ac | +  | +  | +  | 75kDa |
| NAD⁺        | +  | -  | +  | 50kDa |
| Mtb DAc     | -  | +  | +  |     |
Figure 6

Mycobactin MbtA regulation

A

\[ \text{P1} \rightarrow \text{Rv0997} \rightarrow \text{P2} \rightarrow \text{Rv0998} \rightarrow \text{P3} \rightarrow \text{Rv0999} \rightarrow \text{mc}^2\text{6206} \]

- Left flank: 817 bp
- Right flank: 1008 bp

B

\[ \text{P1} \rightarrow \text{res} \rightarrow \text{sacB} \rightarrow \text{hyg}^R \rightarrow \text{res} \rightarrow \text{mc}^2\text{6206} \]

\[ \Delta\text{Rv0998::res-sacB-hyg-res} \]

- Left flank: ~678 bp
- Right flank: ~656 bp

C

\[ \text{P5} \rightarrow \text{Rv1150} \rightarrow \text{P6} \rightarrow \text{Rv1151c} \rightarrow \text{P7} \rightarrow \text{Rv1152} \rightarrow \text{P8} \rightarrow \text{Rv1153} \rightarrow \text{mc}^2\text{6206} \]

- Left flank: 840 bp
- Right flank: 1105 bp

D

\[ \text{P5} \rightarrow \text{res} \rightarrow \text{sacB} \rightarrow \text{hyg}^R \rightarrow \text{res} \rightarrow \text{mc}^2\text{6206} \]

\[ \Delta\text{Rv1151c::res-sacB-hyg-res} \]

- Left flank: ~677 bp
- Right flank: ~730 bp

E

\[ \text{Rv0998} \]

WT Del MW Del WT

\[ \text{Left flank} \rightarrow \text{Right flank} \]

F

\[ \text{Rv1151c} \]

WT Del MW Del WT

\[ \text{Left flank} \rightarrow \text{Right flank} \]
Figure 7

A

B

$\Delta Pat$

$\Delta DAC$

Days

C

D

$\Delta Pat$

$\Delta DAC$

Days
Post-Translational Acetylation of MbtA Modulates Mycobacterial Siderophore Biosynthesis
Olivia Vergnolle, Hua Xu, JoAnn M. Tufariello, Lorenza Favrot, Adel A. Malek, William R. Jacobs, Jr. and John S. Blanchard

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