Reversible acetylation regulates acetate and propionate metabolism in *Mycobacterium smegmatis*

Jennifer D. Hayden,1 Lanisha R. Brown,1 Harsha P. Gunawardena,2 Ellen F. Perkowski,1 Xian Chen2 and Miriam Braunstein1

1Department of Microbiology and Immunology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA
2Department of Biochemistry and Biophysics, School of Medicine, University of North Carolina, Chapel Hill, NC 27599, USA

Carbon metabolic pathways are important to the pathogenesis of *Mycobacterium tuberculosis*, the causative agent of tuberculosis. However, extremely little is known about metabolic regulation in mycobacteria. There is growing evidence for lysine acetylation being a mechanism of regulating bacterial metabolism. Lysine acetylation is a post-translational modification in which an acetyl group is covalently attached to the side chain of a lysine residue. This modification is mediated by acetyltransferases, which add acetyl groups, and deacetylases, which remove the acetyl groups. Here we set out to test whether lysine acetylation and deacetylation impact acetate metabolism in the model mycobacteria *Mycobacterium smegmatis*, which possesses 25 candidate acetyltransferases and 3 putative lysine deacetylases. Using mutants lacking predicted acetyltransferases and deacetylases we showed that acetate metabolism in *M. smegmatis* is regulated by reversible acetylation of acetyl-CoA synthetase (Ms-Acs) through the action of a single pair of enzymes: the acetyltransferase Ms-PatA and the sirtuin deacetylase Ms-SrtN. We also confirmed that the role of Ms-PatA in regulating Ms-Acs regulation depends on cAMP binding. We additionally demonstrated a role for Ms-Acs, Ms-PatA and Ms-SrtN in regulating the metabolism of propionate in *M. smegmatis*. Finally, along with Ms-Acs, we identified a candidate propionyl-CoA synthetase, Ms5404, as acetylated in whole-cell lysates. This work lays the foundation for studying the regulatory circuit of acetylation and deacetylation in the cellular context of mycobacteria.

INTRODUCTION

Lysine acetylation is a reversible post-translational modification (PTM) where an acetyl group is added to the side chain of a lysine residue. Much like phosphorylation, acetylation is an efficient and dynamic way to both positively and negatively regulate protein function. This modification is extensively studied in eukaryotes, especially with regard to its role in histone modifications. So far in prokaryotes, there are only a few examples of proteins regulated by lysine acetylation (Barak et al., 1992; Lima et al., 2011, 2012; Ramakrishnan et al., 1998; Thao et al., 2010; Yan et al., 2008), with the best-characterized acetylated protein being acetyl-CoA synthetase (Acs) (Barak et al., 2004; Starai et al., 2002). However, it is likely that many more bacterial proteins are regulated by lysine acetylation. Proteomic studies identified between 100 and 200 proteins with acetylated lysine residues in *Escherichia coli*, *Salmonella enterica*, *Erwinia amylovora* and *Bacillus subtilis* (Kim et al., 2013; Wang et al., 2010; Wu et al., 2013; Yu et al., 2008; Zhang et al., 2009). These proteins have functions in various processes; however, metabolic enzymes are the largest class of acetylated proteins identified in these studies.

*Mycobacterium tuberculosis* is the causative agent of tuberculosis disease, which is responsible for 1.4 million deaths per year (WHO, 2013). New drugs are needed to control drug-resistant *M. tuberculosis* infections, and a more complete understanding of *M. tuberculosis* biology will guide such efforts. It is clear that the carbon metabolic pathways of *M. tuberculosis* are critical to the growth and persistence of this pathogen in the host (Marrero et al.,

Abbreviations: AUC, area under the curve; FDR, false discovery rate; GNAT, Gcn5-related N-acetyltransferase; LC, liquid chromatography; PTM, post-translational modification; RFU, relative fluorescence units; XIC, extracted ion chromatogram.

Two supplementary figures and a supplementary table are available with the online version of this paper.
Lysine acetylation regulates mycobacterial metabolism

In this study, we investigated the biological consequence of acetylation of Acs in the model mycobacterial species Mycobacterium smegmatis. Acs converts the short chain fatty acid acetate to acetyl-CoA, a central molecule in many metabolic processes including the tricarboxylic acid cycle. While there have been no prior studies of Δacs mutants of mycobacteria, analysis of Δacs mutants of other bacteria demonstrates a role for Acs in enabling growth when a low concentration of acetate is present as the sole carbon source (Castano-Cerezo et al., 2011; Gardner & Escalante-Semerena, 2009; Kumari et al., 1995; Starai et al., 2003). The biological impact of reversible Acs acetylation has been investigated in a small collection of bacteria, with the S. enterica enzyme being one of the best-studied examples (Barak et al., 2004; Castano-Cerezo et al., 2011; Crosby et al., 2010; Gardner et al., 2006). In S. enterica, Acs is acetylated by protein acetyltransferase (Pat), a Gcn5-related N-acetyltransferase (GNAT) (Starai & Escalante-Semerena, 2004). This acetylation inhibits Acs function (Fig. 1a) (Starai & Escalante-Semerena, 2004). In S. enterica, the deacetylase CobB removes the acetyl group, allowing Acs to regain activity (Fig. 1a) (Starai et al., 2002). CobB is a member of the NAD+ -dependent sirtuin, or class III, family of deacetylases. In B. subtilis, there are two deacetylases involved in Acs regulation: a sirtuin SrtN and a Zn-dependent class I deacetylase AcuC (Gardner & Escalante-Semerena, 2009).

Lysine acetylation has been detected in mycobacteria by anti-acetyl lysine Western blot (Nambi et al., 2010). Furthermore, inspection of mycobacterial genomes reveals genes encoding Acs and candidate lysine acetyltransferases and deacetylases, including a large number of predicted GNATs: 25 in M. smegmatis and 20 in M. tuberculosis (UniProt Consortium, 2012). In vitro studies show that the M. smegmatis Pat protein (Ms5458, Ms-PatA) and the M. tuberculosis Pat (Rv0998, Mt-PatA), which share amino acid homology with the S. enterica Pat, can acetylate purified M. tuberculosis Acs (Rs5667, Mt-Acs) (Xu et al., 2011). Mt-PatA has also been referred to as KATmt (Nambi et al., 2013). In vitro studies further demonstrate that acetylated Mt-Acs can be deacetylated by the M. tuberculosis Rv1151c protein or by its M. smegmatis orthologue Ms5175 (Gu et al., 2009; Li et al., 2011a; Xu et al., 2011). We refer to Rv1151c as Mt-SrtN because it is the only predicted sirtuin deacetylase of M. tuberculosis, and we call Ms5175 Ms-SrtN. There are, however, multiple predicted deacetylases in M. smegmatis: two predicted sirtuins, Ms5175 and Ms4620, and a putative Zn-dependent class I deacetylase, Ms0171 (Fig. 1b).

In vitro studies argue that lysine acetylation regulates mycobacterial Acs. However, in the context of the mycobacterial cell the importance of lysine acetylation and deacetylation to Acs and acetate metabolism has never been demonstrated. Here, we carried out a phenotypic analysis of M. smegmatis mutants lacking predicted acetyltransferases and deacetylases, and showed that a single pair of enzymes, Ms-PatA and Ms-SrtN, regulates acetate metabolism, as well as propionate metabolism. Mycobacterial Pat proteins are unusual in containing a cAMP-binding domain, which is required for their function in vitro (Lee et al., 2012; Nambi et al., 2010; Xu et al., 2011). In our study, we confirmed that Ms-PatA requires cAMP binding in cells. Finally, along with Acs, we identified the predicted propionyl-CoA synthetase Ms5404 as being acetylated in M. smegmatis.

**METHODS**

**Bacterial strains and growth conditions.** For genetic manipulations, M. smegmatis mc²155 was grown in 7H9 (Difco) with 0.5% glucose, 0.2% glycerol and 0.1% tyloxapol (Sigma), and grown at 22°C on a gyratory shaker. For initial derepression of Acs, M. smegmatis was grown to early stationary phase (OD₆₀₀ ≈ 0.5) in 7H9 media supplemented with 2% L-glutamate and 0.5% glucose, and then incubated with 1 mM acetate for 24 hours.
37 °C. For growth curves, cultures were grown in M9 media (12.6 mM Na2HPO4, 22 mM KH2PO4, 8 mM NaCl, 19 mM NH4Cl, 2 mM MgSO4, 0.1 mM CaCl2) with 0.1 % tyloxapol. Provided carbon sources included 0.5% glucose and 0.2% glycerol, 2.5 mM sodium acetate, or 2.5 mM sodium propionate. To enumerate c.f.u., cultures were diluted and plated on 7H10 (Difco) with 0.5% glucose, 0.2% glycerol and 0.1% tyloxapol. When needed, antibiotics were used at the following concentrations: 20 μg ml−1 kanamycin, 50 μg ml−1 hygromycin. DH5α E. coli was grown in LB broth (Fisher Scientific) with the following antibiotics when necessary: 40 μg ml−1 kanamycin, 150 μg ml−1 hygromycin, 100 μg ml−1 ampicillin.

**Mutant construction.** *M. smegmatis* mutants were constructed via recombinering (van Kessel & Hatfull, 2008; van Kessel et al., 2008). Briefly, allelic exchange vectors were constructed by cloning 500–800 bp upstream and downstream flanks of the target gene (see Table S1, available in Microbiology Online, for primer information) on either side of a hygromycin-resistance cassette in pMP614 (a kind gift from Martin Pavelka, University of Rochester Medical Center, Rochester, NY, USA). The resulting vector was linearized by digesting with XhoI and KpnI, and was used to transform *M. smegmatis* mc²155 harbouring the multicopy Kan-marked plasmid pJV53, which encodes the phage-derived recombinase genes needed for recombination of linear DNA. Recombinase expression from pJV53 was induced by a 3 h incubation with 0.2 % acetamide. Following induction, electrocompetent cells were prepared and transformed with the linearized allelic exchange vectors (Snapper et al., 1990). All mutants were confirmed by Southern blot analysis (data not shown). Confirmed mutant strains were cured of plasmid pJV53 by growing cultures to saturation without antibiotic and screening for kanamycin-sensitive colonies. To engineer multiple deletions in the same strain, the hygromycin-resistance gene was removed via resovlation recognition sites using pMP854, which expresses TnpR resolvase (a kind gift from Martin Pavelka). pMP854 was later cured from the deletion strains. A complete strain list is found in Table 1 and plasmids are listed in Table 2.

**Complementation vector construction.** To complement mutations, the gene of interest was amplified by PCR (Table S1 for primer information) on either side of a hygromycin-resistance cassette in pMP614 (a kind gift from Martin Pavelka). The resulting vector was linearized by digesting with XhoI and KpnI, and was used to transform *M. smegmatis* mc²155 harbouring the multicopy Kan-marked plasmid pJV53, which encodes the phage-derived recombinase genes needed for recombination of linear DNA. Recombinase expression from pJV53 was induced by a 3 h incubation with 0.2 % acetamide. Following induction, electrocompetent cells were prepared and transformed with the linearized allelic exchange vectors (Snapper et al., 1990). All mutants were confirmed by Southern blot analysis (data not shown). Confirmed mutant strains were cured of plasmid pJV53 by growing cultures to saturation without antibiotic and screening for kanamycin-sensitive colonies. To engineer multiple deletions in the same strain, the hygromycin-resistance gene was removed via resovlation recognition sites using pMP854, which expresses TnpR resolvase (a kind gift from Martin Pavelka). pMP854 was later cured from the deletion strains. A complete strain list is found in Table 1 and plasmids are listed in Table 2.

**Resazurin assays.** *M. smegmatis* growth and viability were monitored using resazurin. Cultures were grown in M9 media with 0.5% glucose, 0.2% glycerol, 0.1% tyloxapol and saturation and then subcultured into new media. At an OD₆₀₀ of 1, cells were washed twice in M9 0.1% tyloxapol (no added carbon source) and diluted to 10⁶ c.f.u. ml⁻¹. Cells (100 μl) were added to 96-well plates containing media as indicated. After 24 h of growth at 37 °C, resazurin (12.5 μg ml⁻¹ final concentration; Sigma) was added and fluorescence with excitation at 530 nm and emission at 590 nm was monitored over time. Values at 10 h post-resazurin addition are reported following normalization to the fluorescence value of the wild-type strain in the same growth media. A Student’s t-test was used to determine if datasets were statistically significantly different. Negative controls always included media with no cells and cells with no added carbon source.

**Western blotting.** Whole-cell lysates of *M. smegmatis* grown in Mueller–Hinton media were prepared by 2–3 passages in a French press. Unlysed cells were removed by centrifugation and protein concentration was determined with a BCA assay (Pierce). Whole-cell lysates were separated by SDS-PAGE. The acetylated lysine primary antibody (Cell Signalling) was used at a 1:2500 dilution and was incubated with either BSA or acetylated BSA (100 μg ml⁻¹ final concentration) for 1 h on ice before addition to membranes. IRDye 800CW goat anti-rabbit polyclonal antibody (LI-COR) was used as the secondary antibody at a 1:15,000 dilution. The HA antibody (Covance) was used at a 1:20,000 dilution with the IRDye 680 T goat anti-mouse secondary antibody (LI-COR) used at a 1:20,000 dilution. Western blot results were visualized with an Odyssey fluorescence imaging system (LI-COR). Quantification of band densitometry was performed using Image J software (W. S. Rasband, NIH, Bethesda, MD, USA, 1997–2012; http://imagej.nih.gov/ij/). As a control for protein loading, a polyclonal SigA antibody (a kind gift from Murty Madiraju, The University of Texas Health Science Center, Tyler, TX, USA) was used at 1:20,000.

**In-gel trypsin digestion for MS analysis.** For both the wild-type and ΔdeAc mutant, 7 mg whole-cell lysate protein was separated on a 10 % SDS-PAGE gel (Bio-Rad). The protein bands were visualized by Coomassie blue R-250 staining (Bio-Rad), and gel slices corresponding to proteins 60–75 kDa in size were excised. Digestion of the excised gel slices was performed with 20 μg sequeding grade trypsin ml⁻¹ in 25 mM ammonium bicarbonate as described elsewhere (Collins et al., 2008).

**Liquid chromatography (LC)-MS-MS.** Identification of proteins was done using reversed-phase LC-MS-MS analysis on a 2D-nanoLC Ultra system (Eksigent) coupled to an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). The Eksigent system was configured to trap and elute peptides via a sandwiched injection of 250 fmol sample. The trapping was performed on a 3 cm long,

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>mc²155</td>
<td>Wild-type <em>M. smegmatis</em></td>
<td>Snapper et al. (1990)</td>
</tr>
<tr>
<td>JDH032</td>
<td>mc¹155 Δms-ac3 (ms6179)</td>
<td>This study</td>
</tr>
<tr>
<td>JDH008</td>
<td>mc¹155 Δms-srtN (ms5175)</td>
<td>This study</td>
</tr>
<tr>
<td>JDH015</td>
<td>mc¹155 Δms6260</td>
<td>This study</td>
</tr>
<tr>
<td>JDH037</td>
<td>mc¹155 Δms0171</td>
<td>This study</td>
</tr>
<tr>
<td>JDH043</td>
<td>mc¹155 Δms-srtN Δms6260 Δms0171</td>
<td>This study</td>
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<td>JDH050</td>
<td>mc¹155 Δms-patA</td>
<td>This study</td>
</tr>
<tr>
<td>JDH051</td>
<td>mc¹155 Δms-srtN Δms6260 Δms0171 Δms-patA</td>
<td>This study</td>
</tr>
</tbody>
</table>

**Table 1.** Bacterial strain list
Table 2. Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Antibiotic marker</th>
<th>Source</th>
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<tr>
<td>pMV261.kan</td>
<td>Mycobacterial multicopy vector; hsp60-dependent promoter</td>
<td>Kan^R</td>
<td>Stover et al. (1991)</td>
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<tr>
<td>pJS577</td>
<td>HA.tag cloned into pMV261.kan</td>
<td>Kan^R</td>
<td>Glickman et al. (2000)</td>
</tr>
<tr>
<td>pJV53</td>
<td>Multicopy plasmid encoding phage Che9c 60 and 61 genes under acetamide-inducible promoter</td>
<td>Kan^R</td>
<td>van Kessel &amp; Hatfull (2008); van Kessel et al. (2008)</td>
</tr>
<tr>
<td>pMP614</td>
<td>res-Hyg-res plasmid for constructing allelic exchange vectors</td>
<td>Hyg^R</td>
<td>Kind gift from Martin Pavelka</td>
</tr>
<tr>
<td>pMP854</td>
<td>trpR expressing plasmid used to remove HygR marker from mutant strains</td>
<td>Kan^R</td>
<td>Kind gift from Martin Pavelka</td>
</tr>
<tr>
<td>pH33</td>
<td>M. smegmatis ms-acs (ms6179) cloned into pMV261</td>
<td>Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pEP104</td>
<td>M. tuberculosis ms-srtN (rv1151c) cloned into pJS577</td>
<td>Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pH40</td>
<td>M. smegmatis ms-patA (ms5458) cloned into pMV306.kan</td>
<td>Kan^R</td>
<td>This study</td>
</tr>
<tr>
<td>pHJ51</td>
<td>M. smegmatis ms-patA (ms5458) HA-tagged and cloned into pMV306.kan</td>
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<td>This study</td>
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<tr>
<td>pHJ52</td>
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<td>Kan^R</td>
<td>This study</td>
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</tbody>
</table>

100 μm i.d. C18 column, while elution was performed on a 15 cm long, 75 μm i.d., 5 m, 300 A particle ProteoPep II integraFrit C18 column (New Objective). Analytical separation of the tryptic peptides was achieved with a 120 min linear gradient of 2–10% buffer B at 200 nL min⁻¹, where buffer A was an aqueous solution of 0.1% formic acid and buffer B was a solution of 0.1% formic acid in acetonitrile.

Mass spectrometric data acquisition was performed in a data-dependent manner via high-energy collision dissociation of the top 10 most abundant ions a hybrid LTQ-Orbitrap. Velos mass spectrometer mass spectra were processed, and peptide identification was performed using the Andromeda search engine found in MaxQuant software version 2.2.1 (Max Planck Institute, Germany). All protein database searches were performed against the M. smegmatis protein sequence database downloaded from PATRIC (Pathosystems Resource Integration Center) (http://patricbrc.org; Virginia Bioinformatics Institute, Blacksburg, VA, USA) (Gillespie et al., 2011).

It should be noted that the PATRIC annotation for Ms-Acs differs from the National Center for Biotechnology Information annotation (YP_890399.1) and was used as a reference in this study because the molecular mass of Ms-Acs observed on our Western blots corresponds with the PATRIC annotation.

Peptides were identified using a target-decoy approach with a false discovery rate (FDR) of 1% (Bantscheff et al., 2012). A precursor ion mass tolerance of 100 p.p.m. was used for the first search that allowed for m/z retention time recalibration of precursor ions that were then subjected to a main search using a precursor ion mass tolerance of 5 p.p.m. and a product ion mass tolerance 0.5 Da. Search parameters included up to two missed cleavages at KR on the sequence (Cox & Mann, 2011), and lysine acetylation and methionine oxidation as dynamic PTMs. All acetylations representing a differential modification of 42.037 Da on lysines were unambiguously localized to specific residues using the built-in PTM score with a probability of >0.99.

Acetyl peptide and protein quantification. Label-free quantification was based on peak area (Bondarenko et al., 2002; Chelius & Bondarenko, 2002; Gunawardena et al., 2011). The measured area under the curve (AUC) of m/z and retention time aligned extracted ion chromatogram (XIC) of a peptide was performed via the label-free quantification module found in MaxQuant (version 1.2.2.5). Protein level quantification was performed using unique and razor peptide features corresponding to identifications filtered with a peptide FDR of 1%, and protein FDR of 5% and PTM FDR 1%. Data processing was performed using Peseus (version 1.2.0.17) (Max Planck Institute, Germany) where relative quantification changes between samples were reported as fold-change differences. MaxQuant acetylated peptide-level quantification values were verified manually by integrating the AUC of the XIC using the Genesis peak detection and integration algorithm found in Xcalibur software (Thermo Scientific).

RESULTS

Acs is required for acetate metabolism in M. smegmatis

In other bacteria, Acs is required for growth on low levels of acetate as a sole carbon source and acetylation negatively regulates this activity (Castañó-Cerezo et al., 2011; Gardner & Escalante-Semerena, 2009; Kumari et al., 1995; Starai et al., 2003). Before investigating the impact of lysine acetylation on Acs in mycobacteria, we first needed to establish a role for Ms-Acs in enabling growth of M. smegmatis on low levels of acetate. As there are no prior reports of Δacs mutants of mycobacteria, we began by constructing an M. smegmatis mutant with a deletion of the annotated ms-acs gene (ms6179). Wild-type and the Δms-acs mutant were grown in M9 minimal media containing either glucose and glycerol or acetate for carbon sources. At various time points, culture aliquots were serially diluted and plated on glucose and glycerol containing agar to determine the number of c.f.u. Although the c.f.u. ml⁻¹ of the Δms-acs mutant was comparable to wild-type in glucose and glycerol media over time, the mutant exhibited a growth defect in media with acetate as the sole carbon source (Fig. 2a, b). The acetate growth phenotype of the Δms-acs mutant was complemented when the M. smegmatis ms-acs gene was expressed from a plasmid, which confirmed that this phenotype is due to the loss of Ms-Acs.

As another way to test for an acetate metabolism defect we employed a resazurin reduction assay (McNerney et al., 2011).
Fig. 2. Ms-Acs is required for acetate metabolism in *M. smegmatis*. (a) Growth curve of wild-type (mc²155, pMV261), Δms-acs (JDH032, pMV261) and Δms-acs complemented with ms-acs (JDH032, pJH33) strains in glucose/glycerol media. Aliquots from liquid cultures were removed at the indicated time points and plated to enumerate c.f.u. ml⁻¹. (b) Growth curve of wild-type, Δms-acs and Δms-acs complemented with ms-acs strains in acetate media. Experiment as described in (a). *P* value of <0.01 when comparing the wild-type and ms-acs strains. (c) Resazurin reduction in glucose/glycerol media. Liquid cultures of wild-type, Δms-acs and Δms-acs complemented with ms-acs strains were grown in 96-well plates as described in Methods. After 24 h, resazurin was added and relative fluorescence units (RFU) were measured over the next 20 h. (d) Resazurin reduction in acetate media, as described for (c). (e) The 96-well plate 10 h after resazurin was added. Pink colour indicates metabolically active cells, while blue shows low resazurin reduction and low metabolic activity. (f) Quantification of resazurin fluorescence for cells grown in glucose/glycerol (white bars) and acetate (black bars). The fluorescence value at 10 h after resazurin addition was normalized to that of the wild-type in each growth condition. *P* value of <0.01 when compared to wild-type fluorescence in the same media. For (a–d) and (f), error bars denote the sd of three replicates.
Resazurin is a blue compound that is reduced by metabolically active cells. When reduced, resazurin is converted to a pink fluorescent product that can be quantified to report on metabolic activity and cell number. Strains were grown in a 96-well plate in either glucose and glycerol or acetate-containing media. After 24 h incubation, resazurin was added and 10 h later visually inspected. In glucose and glycerol media, the wild-type, Δms-acs mutant and complemented ms-acs strain all showed robust resazurin reduction, as indicated by the pink colour (Fig. 2e). In acetate media, the wild-type and complemented strain also reduced resazurin, although the pink colour was not as strong as when the same strains were grown with glucose and glycerol, which are known to be better carbon sources. However, wells containing the Δms-acs mutant in acetate media maintained a blue colour, indicating reduced metabolic activity for the mutant (Fig. 2e). We also measured resazurin fluorescence over the course of 20 h (Fig. 2c, d). All strains converted resazurin at an equal rate in glucose and glycerol media. In acetate media, the wild-type and complemented strain reduced resazurin while the Δms-acs mutant showed very little to no resazurin reduction. In fact, the resazurin reduction of the Δms-acs mutant in acetate was comparable to what is seen when M. smegmatis is incubated with no carbon source (data not shown). For simplicity, we decided to report these data at a single time point of 10 h post-resazurin addition with the fluorescence value of each strain set relative to that of wild-type in the same carbon source (Fig. 2f). Taken together, the c.f.u. plating and resazurin assays demonstrated that, like in other bacteria, Ms-Acs is required for acetate metabolism and growth in acetate media.

SrtN deacetylase regulates acetate metabolism in M. smegmatis

Having established function for Ms-Acs, we tested whether Ms-Acs and acetate metabolism are regulated by reversible lysine acetylation (Fig. 1a). If Ms-Acs is inhibited by lysine acetylation, there must exist at least one deacetylase that removes the acetyl group to activate Ms-Acs. Inspection of the M. smegmatis genome revealed three potential deacetylases. Ms-SrtN (Ms5175) and Ms4620 have homology to the bacterial sirtuins CobB of S. enterica and SrtN of B. subtilis (Fig. 1b and Fig. S1). Ms-SrtN from M. smegmatis is the orthologue of Mt-SrtN from M. tuberculosis, with the two proteins sharing 81% amino acid similarity. Ms4620 has no obvious M. tuberculosis orthologue. Ms0171 is a potential class I Zn-dependent deacetylase that is similar to the class I deacetylase AcuC of B. subtilis (Gardner & Escalante-Semerena, 2009) (Fig. 1b). There is no evident M. tuberculosis orthologue of Ms0171.

To identify the deacetylase(s) that acts on acetylated Ms-Acs in M. smegmatis, we deleted the genes encoding the three deacetylases individually and in combination. We predicted that in the absence of the responsible deacetylase, Ms-Acs would become locked in an acetylated (inactive) state resulting in an inability of M. smegmatis to utilize acetate as the sole carbon source. A Δms-srtN mutant behaved like wild-type M. smegmatis when grown in glucose and glycerol media, as measured by resazurin reduction (Fig. 3, white bars). However, in acetate media the Δms-srtN mutant exhibited significantly reduced metabolic activity, which is consistent with a function of Ms-SrtN in deacetylating Ms-Acs (Fig. 3, black bars). The acetate phenotype of the Δms-srtN mutant was complemented by providing a copy of the M. tuberculosis srtN (mt-srtN) gene. Together, these experiments demonstrated that Ms-SrtN is required for M. smegmatis to optimally metabolize acetate and further showed that Mt-SrtN of M. tuberculosis can also carry out this function. The Δms-srtN mutant phenotype was statistically significant compared to wild-type; however, it is noteworthy that this phenotype was not as severe as that of the Δms-acs mutant.

We also tested deletion mutants lacking ms4620 or ms0171. Both of these mutants behaved like wild-type in glucose and glycerol media as well as in acetate media, indicating that on their own neither of these proteins is required for Acsc deacetylation (Fig. 3). It remained a possibility, however, that the proteins have redundant deacetylase functions. To address this possibility, we constructed an unmarked mutant lacking all three M. smegmatis deacetylases, Δms-srtN Δms4620 Δms0171. We refer to this triple deacetylase mutant as ΔdeAc. The ΔdeAc mutant exhibited a phenotype similar to the single Δms-srtN mutant when grown in acetate. Once again, introduction of the mt-srtN gene was sufficient to restore acetate metabolism to the ΔdeAc mutant. These results are consistent with negative regulation of Ms-Acs by lysine acetylation and with the function of Ms-SrtN and Mt-SrtN being to deacetylate mycobacterial Acs and enable acetate metabolism.

PatA acetyltransferase regulates acetate metabolism in M. smegmatis

The M. smegmatis PatA (Ms5458, Ms-PatA) has been shown to acetylate Mt-Acs in vitro (Nambi et al., 2010; Xu et al., 2011). However, the in vitro studies did not test any of the 24 other putative GNATs in M. smegmatis that could possibly act on Ms-Acs (UniProt Consortium, 2012). To determine if Ms-PatA is the sole acetyltransferase that regulates Ms-Acs in M. smegmatis, we constructed Δms-pata M. smegmatis mutants in the wild-type and the ΔdeAc mutant background. In the model where lysine acetylation of Ms-Acs prevents the enzyme from converting acetate to acetyl-CoA, we predicted that a Δms-pata mutation in a wild-type strain would either enhance or have no effect on acetate metabolism. However, if Ms-PatA is the only acetyltransferase that acts on Ms-Acs then a Δms-pata mutation should rescue the acetate phenotype of the ΔdeAc mutant. In other words, if Ms-Acs is not acetylated in the
revealed that Ms-PatA expression from a plasmid in the
Interestingly, the Ms-PatA complementation experiments
statistically significant.

In the absence of Ms-SrtN deacetylase, the Ms-PatA deletion was introduced into the triple deacetylase mutant, creating the quadruple mutant ΔdeAc Δms-patA, we found that it rescued the ΔdeAc acetate phenotype (Fig. 4). The effect of the Δms-patA deletion in the ΔdeAc mutant background could be complemented when ms-patA was provided on a plasmid, resulting in the return of an acetate metabolism defect. These results are consistent with Ms-PatA being the only acetyltransferase regulating Ms-Acs in M. smegmatis. The Δms-patA deletion in a wild-type background had no effect on M. smegmatis in glucose and glycerol media (Fig. 4). In acetate media the Δms-patA mutant consistently had slightly higher metabolic activity than wild-type, although this effect was not statistically significant.

Interestingly, the Ms-PatA complementation experiments revealed that Ms-PatA expression from a plasmid in the ΔdeAc mutant caused a more severe acetate phenotype than exhibited by the ΔdeAc mutant with Ms-PatA expressed from its native chromosomal location (compare the second and fourth black bars of Fig. 4). Although we have not quantified Ms-PatA levels, this difference could reflect higher expression off the complementing plasmid’s hsp60 promoter than from the chromosomal ms-patA locus.

**Ms-PatA activity requires cAMP binding in M. smegmatis**

Mycobacterial Pat proteins are unique in that they contain an N-terminal cAMP-binding domain, and Ms-PatA requires cAMP for acetyltransferase activity in vitro (Nambi et al., 2010; Xu et al., 2011). In order to test if cAMP binding is also necessary for Ms-PatA activity in the context of the M. smegmatis cell, we tested a Ms-PatA protein in which the arginine at position 95, which is required for cAMP binding in vitro, was substituted with a lysine (Ms-PatA R95K) (Nambi et al., 2010). Unlike the plasmid expressing Ms-PatA, when a plasmid expressing Ms-PatA R95K was introduced into the ΔdeAc Δms-patA strain, it failed to complement the Δms-patA mutation. The difference between the level of acetate metabolism of the ΔdeAc Δms-patA mutant expressing Ms-PatA compared to the strain expressing Ms-PatA R95K was statistically significant (Fig. 5a). To eliminate the possibility that the R95K substitution in Ms-PatA compromised protein stability, these experiments were performed with HA-tagged Ms-PatA and Ms-PatA R95K, and Western blotting confirmed equivalent levels of protein in the strains (Fig. 5b). These experiments validate the importance of cAMP binding to Ms-PatA, reported in vitro (Lee et al., 2012; Nambi et al., 2010; Xu et al., 2011), to Ms-PatA function in the cellular context of intact M. smegmatis.

**Acetylated Ms-Acs is present in M. smegmatis whole-cell lysates**

To examine Ms-Acs acetylation in cells more directly we used an antibody to acetylated lysine (Cell Signalling) and performed Western blotting of whole-cell lysates of M. smegmatis wild-type and the above-mentioned mutants (Fig. 6a). In wild-type M. smegmatis, a band close to the predicted molecular mass of Ms-Acs (69 kDa) was recognized by the acetylated lysine antibody. This band was absent in the Δms-acs mutant, but was present when a plasmid expressing Ms-Acs was introduced into the
mutant, which confirmed the identity of this species as Ms-Acs. To establish the specificity of the antibody for acetylated lysines, we repeated the Western blotting in the presence of acetylated BSA (Fig. 6b). Including acetylated BSA led to a significant decrease in the intensity of the Acs band, confirming that the antibody recognition of Ms-Acs reflects its acetylation. Western blotting in the presence of acetylated BSA also revealed an unidentified protein that was non-specifically recognized by the antibody (Fig. 6b, marked by the plus sign).

We next determined the effects of the deacetylase and ms-patA mutations on Ms-Acs acetylation. Based on the acetate phenotypes of the deacetylase mutants, which prevented acetate metabolism presumably as a result of trapping Ms-Acs in an acetylated state, we expected the Δms-srtN and ΔdeAc mutants to show increased levels of acetylated Ms-Acs. Indeed, in both the Δms-srtN and ΔdeAc mutant strains, an increase in the amount of acetylated Ms-Acs was observed compared to the wild-type strain (Fig. 6). We also evaluated complemented strains in

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**Fig. 4.** Ms-PatA regulates acetate metabolism in *M. smegmatis*. Resazurin reduction was fluorescently monitored and quantified as in Fig. 2(f). The strains included were wild-type (mc²155, pMV261), ΔdeAc (JDH043, pMV261), ΔdeAc Δms-patA (JDH051, pMV261), ΔdeAc Δms-patA complemented with *ms-patA* (JDH051, pJH40), Δms-patA (JDH050, pMV261), and Δms-patA complemented with *ms-patA* (JDH050, pJH40). White bars indicate cultures with glucose/glycerol and black bars indicate cultures with acetate as the sole carbon source. *P < 0.01 when compared to wild-type fluorescence in the same media.

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**Fig. 5.** cAMP binding is required for Ms-PatA activity in *M. smegmatis* cells. (a) Resazurin reduction for cultures grown with acetate as the sole carbon source was fluorescently monitored and quantified as in Fig. 2(f). The strains included were wild-type (mc²155, pMV261), Δms-acs (JDH032, pMV261), ΔdeAc (JDH043, pMV261), ΔdeAc Δms-patA (JDH051, pMV261), ΔdeAc Δms-patA complemented with *ms-patA-HA* (JDH051, pJH51), and ΔdeAc Δms-patA complemented with *ms-patA R95K-HA* (JDH051, pJH52). *P < 0.01 when compared to wild-type. †P < 0.01 when comparing ΔdeAc Δms-patA + ms-patA-HA and ΔdeAc Δms-patA + ms-patA R95K-HA. (b) Western blot of whole-cell lysates of ΔdeAc Δms-patA complemented with *ms-patA-HA* (JDH051, pJH51) and ΔdeAc Δms-patA complemented with *ms-patA R95K-HA* (JDH051, pJH52) probed with anti-HA antibody. M, molecular mass marker.
which *mt-srtN* was introduced on a plasmid into the deacetylase mutant strains. Interestingly, in these *mt-srtN* complemented strains the level of acetylated Ms-Acs only decreased slightly, if at all (Fig. 6c). Because these *mt-srtN* complemented strains were able to metabolize acetate like wild-type (Fig. 3), this result suggested that the reduction in Ms-Acs acetylation needed for acetate metabolism is small.

Next we examined the levels of acetylated Ms-Acs in the Δ*ms-patA* background. In fact, Western blotting revealed undetectable levels of acetylated Ms-Acs when *ms-patA* was deleted (Fig. 6). In complementing strains in which *ms-patA* was provided on an integrating plasmid, acetylated Ms-Acs was again detected. These data fit the model whereby, at least under the conditions tested, Ms-PatA is the sole acetyltransferase that acetylates Ms-Acs.

The Western blots also revealed another acetylated protein running at a slightly lower molecular mass (approx. 65 kDa) than Ms-Acs (Fig. 6a, marked by the arrowhead). This additional acetylated protein was not observed in all

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**Fig. 6.** Mycobacterial SrtN and PatA affect Ms-Acs acetylation. (a) Western blot of whole-cell lysates of indicated strains harbouring empty vectors (EV) or complementing plasmids (genes as indicated). Molecular mass marker band sizes are indicated on the left. The blot was probed with anti-acetyl-lysine antibody in the presence of BSA and shows all proteins detected by the antibody. The arrow points to the specific band corresponding to Ms-Acs, the plus sign indicates a non-specific band and the arrowhead indicates a specifically acetylated unknown protein. (b) Blot probed with anti-acetyl-lysine antibody in the presence of acetylated BSA. (c) Quantification of acetylated Ms-Acs in the strains from (a), performed in triplicate. Each sample was standardized by comparing to the amount of SigA (data not shown). Triplicate samples were then averaged and normalized to the levels of acetylated Ms-Acs in the wild-type strain.
strains, but it was specifically detected in the deacetylase mutants (Δms-srtN and ΔdeAc) and in strains expressing ms-patA from a plasmid. Thus, this band appeared to correspond to another protein that can be acetylated by Ms-PatA and deacetylated by Ms-SrtN.

**Acetylated Ms-Acs and Ms5404 peptides are detected in M. smegmatis whole-cell lysates**

To help identify the acetylated proteins revealed by Western blot, we employed quantitative MS. Whole cell lysates from the wild-type and ΔdeAc strains (two biological replicates of each) were separated by SDS-PAGE and gel slices spanning proteins approximately 60–75 kDa were excised, subjected to trypsin digestion and analysed by LC-MS-MS. Peptide sequences were identified using Andromeda (MaxQuant) and quantification was performed using label-free quantification to measure the AUC of the XIC of peptides (Cox et al., 2011). The analysis unambiguously identified an acetylated Ms-Acs peptide, which was acetylated at lysine residue 589 in the peptide SGK(Ac)IMR (Fig. S2 available with the online version of this paper). This acetylated lysine in Ms-Acs corresponds to the site of acetylation in the S. enterica and B. subtilis Acs proteins. The acetylated Acs peptide was, as expected, more abundant (97.5-fold higher) in the ΔdeAc mutant compared to the wild-type strain (Table 3). In contrast, relatively equal levels of the Ms-Acs protein, quantified with at least five unique peptides, were identified in the ΔdeAc mutant and wild-type strains.

The 67kD Ms5404 protein was also identified as an acetylated protein by the MS analysis. Ms5404 is a predicted propionyl-CoA synthetase and the site of acetylation was unambiguously localized at lysine-586 in the identified peptide SGK(Ac)ILR (Fig S2). Notably, a acetylation was unambiguously localized at lysine-586 in predicted propionyl-CoA synthetase and the site of acetylated protein by the MS analysis. Ms5404 is a 67kD protein was also identified as an acetylated at the corresponding lysine (Garrity et al., 2007). Interestingly, the acetylated Ms5404 peptide was only detected in the ΔdeAc samples while the relative levels of Ms5404 protein, quantified with at least five unique peptides, were similar in the ΔdeAc mutant and wild-type samples (Table 3). These results indicated that Ms5404 is another lysine acetylated protein of M. smegmatis. Its size of 67 kDa and our identification of acetylated peptides only in the ΔdeAc samples makes it a leading candidate for being the acetylated protein observed by Western blot in the ΔdeAc mutant, but not in wild-type (Fig. 6a).

**Ms-Acs, Ms-SrtN and Ms-PatA also regulate propionate metabolism**

Our finding that another acetylated protein of M. smegmatis is Ms5404, a putative propionyl-CoA synthetase, along with the fact that propionate can be a substrate for Mt-Acs in vitro (Li et al., 2011a), suggested that propionate metabolism in M. smegmatis may also be regulated by lysine acetylation. Therefore, we went on to evaluate the deacetylase and acetyltransferase mutants in media with propionate as the sole carbon source. The Δms-srtN and ΔdeAc mutants had reduced metabolic activity when propionate was the sole carbon source (Fig. 7). Similar to what was seen with acetate, addition of the Δms-patA mutation into the ΔdeAc background rescued propionate metabolism. All the propionate phenotypes were complemented when the appropriate genes were provided on a plasmid. These results indicated that Ms-SrtN and Ms-PatA additionally regulate propionate metabolism in M. smegmatis. While the Δms-acs mutant also had a phenotype, the metabolism defect of the mutant in propionate was not as complete as seen in acetate (compare Fig. 3 and Fig. 7), suggesting there could be another protein acting as a propionyl-CoA synthetase in the cell, with Ms5404 being a leading candidate.

**DISCUSSION**

In comparison to eukaryotic cells, lysine acetylation in bacteria is a more recently recognized phenomenon that has so far only been studied in a small number of bacterial species. Acs is the best-studied example of a bacterial protein regulated by lysine acetylation, and previous in vitro studies demonstrate the ability of mycobacterial Pat and SrtN enzymes to acetylate and deacetylate purified Acs, respectively (Gu et al., 2009; Li et al., 2011a; Xu et al., 2011). Published work also shows that the Pat protein of Mycobacterium bovis BCG is necessary for growth on

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<th>Table 3. Label-free quantification values obtained for Ms-Acs and Ms5404 proteins and their detectable acetylated peptides</th>
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<td><strong>Label-free AUC</strong></td>
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ND, peptides that had no detectable XIC-AUC in label-free quantification, but were assigned a value equal to a base line noise level of 1.0 × 10⁵ in order to calculate a fold-change value.
propionate as a sole carbon source (Nambi et al., 2013). However, none of the past studies demonstrated the biological significance of the Acs acetylation/deacetylation regulatory circuit on acetate metabolism in mycobacteria. In the present study, we demonstrated a role for the lysine acetyltransferase Ms-PatA and deacetylase Ms-SrtN in regulating Ms-Acs and acetate metabolism in the context of the cell. Thus, the results reported here are significant in providing critical validation of the data obtained by in vitro studies with purified proteins. We also confirmed that the cAMP binding of Ms-PatA is important for its function and we identified an additional example of an acetylated protein, Ms5404, in *M. smegmatis*.

**Specificity of Ms-SrtN and Ms-PatA enzymes**

There are several putative lysine acetyltransferases and deacetylases encoded by the *M. smegmatis* genome. It was a strong possibility that acetylation in mycobacteria would involve multiple modifying enzymes and prove more complicated than observed in vitro. In fact, in *B. subtilis*, two deacetylases are required for Acs deacetylation and growth on acetate (Gardner & Escalante-Semerena, 2009). However, we found only one deacetylase, Ms-SrtN, acted on acetylated Ms-Acs. Ms-SrtN is the orthologue of the sole predicted deacetylase in *M. tuberculosis*, Mt-SrtN. When complementing the Δms-srtN mutant phenotype we used a plasmid expressing Mt-SrtN, which demonstrated conservation of function of the *M. tuberculosis* and *M. smegmatis* SrtN proteins. The functions of the other two putative *M. smegmatis* deacetylases are still in question. While there was no evidence for either Ms4620 or Ms0171 acting on Ms-Acs, they may deacetylate other substrates.

Given the large number of putative GNAT-type acetyltransferases (UniProt Consortium, 2012), we also thought it possible that more than one of these proteins would act on Ms-Acs, but that was not the case. Thus, there appears to be a high level of substrate specificity for the Ms-SrtN and Ms-PatA pair.

Data in this study are believed to be the first to show an acetate phenotype for mycobacterial deacetylase mutants. The acetate phenotypes of the deacetylase mutants (Δms-srtN or ΔdeAc) were reproducible, complementable and statistically significant, but they were not as severe as the phenotype of the Δms-acs mutant. In fact, until we turned to the resazurin assay to measure acetate metabolism it was difficult to observe a phenotype, which may be why there were no earlier reports of deacetylation regulating acetate metabolism in intact cells. The more moderate phenotype of the deacetylase mutants suggested that there still existed some active unacetylated Ms-Acs in these strains. This could be due to the presence of an unidentified deacetylase. However, we think it more likely that the ΔdeAc acetate phenotype is due to newly synthesized Ms-Acs that had yet to be acetylated in the cell, possibly as a consequence of limited amount or acetylation efficiency of Ms-PatA.

Support for this idea comes from experiments where the acetate defect of the ΔdeAc mutant was enhanced by providing an exogenous copy of Ms-PatA on a single copy vector (Fig. 4, compare ΔdeAc and ΔdeAc Δms-patA Δms-srtN Δms-acs + ms-patA). In addition, overexpressing Ms-PatA from a multi-copy vector in a wild-type background prevented *M. smegmatis* growth on acetate (data not shown). These experiments suggested that the level of Ms-PatA influences the amount of acetylated inactive Ms-Acs in the cell.
An additional metabolic process regulated by Ms-Acs, Ms-SrtN and Ms-PatA

Our data also revealed a role for Ms-Acs, Ms-SrtN and Ms-PatA in regulating propionate metabolism in \textit{M. smegmatis} (Fig. 7). \textit{In vitro} studies show Mt-Acs to be able to utilize propionate, in addition to acetate, to generate CoA derivatives (Li et al., 2011a; Xu et al., 2011). However, it is likely that Ms-Acs is not the sole propionyl-CoA synthetase in \textit{M. smegmatis} because the \textit{Δms-acs} phenotype in propionate was not complete. The other protein we identified by MS as being lysine acetylated, Ms5404, is the leading candidate for being an additional propionyl-CoA synthetase that could enable propionate utilization by \textit{M. smegmatis}. In \textit{S. enterica}, both Acs and a protein called PrpE have propionyl-CoA synthetase activity but, in this case, both proteins need to be absent in order to observe a phenotype on propionate (Horswill & Escalante-Semerena, 1999).

In our study the \textit{Δms-patA} mutation in a wild-type background did not exhibit a defect in propionate metabolism, although it did rescue the \textit{Δdeac} phenotype. This differs from the recent report of an \textit{M. bovis} BCG \textit{Δpat} mutant (\textit{ΔKATb)} being defective for growth in propionate (Nambi et al., 2013). The BCG \textit{Δpat} mutant phenotype was interpreted as resulting from a build-up of Acs-generated toxic propionyl-CoA metabolites. Interestingly, in this study, it was also shown that the BCG Acs can be propionylated by PatA and depropionylated by SrtN \textit{in vitro} (Nambi et al., 2013), suggesting that acetylation and depropionylation may provide overlapping levels of regulation in mycobacteria. Because propionyl-CoA metabolite accumulation can also be toxic to \textit{M. smegmatis} (Upton & McKinney, 2007), the different results we obtained with the \textit{Δms-patA} mutant may be accounted for by differences in experimental conditions and/or the specific metabolic pathways operating in each species to prevent propionyl-CoA build-up (Rhee et al., 2011). It also remains possible that the phenotype of the BCG \textit{ΔpatA} mutant reflects PatA regulated proteins other than Acs since the phenotype of a BCG \textit{Δacs} mutant in propionate is unknown. Nonetheless, from the studies of \textit{ΔpatA} mutants in \textit{M. smegmatis} and BCG it is evident that propionate metabolism in mycobacteria is regulated by PatA.

Expanding our understanding of mycobacterial metabolic regulation

Until recently, the only mode of metabolic regulation reported in mycobacteria was transcriptional regulation of metabolic genes (Datta et al., 2011; Schnappinger et al., 2003; Timm et al., 2003). Interestingly, both the mycobacterial SrtN and PatA enzymes themselves are regulated by different cellular signals. Transcript levels of the \textit{patA} gene of \textit{M. bovis} BCG are reported to be regulated by propionate and SDS (Nambi et al., 2013). In addition, cAMP binding to Mt-PatA causes major conformational changes (Lee et al., 2012) and increases the acetylation activity of both Ms-PatA and Mt-PatA proteins \textit{in vitro} (Nambi et al., 2010; Xu et al., 2011). Our data extend these \textit{in vitro} results in showing that cAMP binding is also necessary for PatA activity in \textit{M. smegmatis} cells. In \textit{M. tuberculosis}, cAMP levels are regulated by several conditions associated with infection (Bai et al., 2011). Interestingly, cAMP also regulates the PatZ lysine acetyltransferase of \textit{E. coli}, but it does so by a different mechanism involving transcriptional regulation (Castaño-Cerezo et al., 2011).

On the deacetylase side of the equation, the mycobacterial sirtuin deacetylases Ms-SrtN and Mt-SrtN are shown to be NAD$^+$-dependent deacetylases \textit{in vitro} (Gu et al., 2009; Nambi et al., 2013; Xu et al., 2011). NAD$^+$ levels reflect the energy status of the cell. In addition to cAMP and NAD$^+$, the level of acetyl-CoA, which is used in the acetylation reaction and is another reflection of the carbon and energy status of the cell, will also influence the amount of acetylation that occurs. Consequently, reversible acetylation by the mycobacterial PatA and SrtN pair provides an interesting example of how multiple signals and regulatory networks can be integrated to achieve metabolic regulation.

There are likely to be many mycobacterial proteins regulated by lysine acetylation besides Ms-Acs and Ms5404. In other studies, a universal stress protein, Ms4207, of \textit{M. smegmatis} was reported to be acetylated by Ms-PatA (Nambi et al., 2010) and FadD13 was identified as acetylated in \textit{M. bovis} BCG cells (Nambi et al., 2013). However, the biological significance of Ms4207 or FadD13 acetylation has yet to be shown. There are also multiple FadD proteins (fatty acyl-CoA ligases) from \textit{M. tuberculosis} shown to be acetylated and deacetylated by PatA and SrtN with purified proteins \textit{in vitro} (Nambi et al., 2013). There is additionally a report of a \textit{Δms-srtN} mutant having a defect in DNA double strand break repair, although this phenotype has not been linked to deacetylation of any substrates (Li et al., 2011b). In the future it will be important to identify the full acetylome of mycobacterium species, especially \textit{M. tuberculosis}.

Reversible acetylation is a strong candidate for dynamically controlling metabolic pathways and enabling \textit{M. tuberculosis} to quickly adapt to the changing host environment. By showing here that the regulatory circuit of Acs acetylation and deacetylation by PatA and SrtN enzymes holds true in \textit{M. smegmatis}, this work opens the door to studying reversible acetylation and how it integrates regulatory signals in the cellular environment of mycobacteria.

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Lysine acetylation regulates mycobacterial metabolism


