Identification of the dehydratase component of the mycobacterial mycolic acid-synthesizing fatty acid synthase-II complex

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Mycolic acids are vital components of the Mycobacterium tuberculosis cell wall and are essential for survival. While most components of the fatty acid synthase-II (FAS-II) enzymic machinery that synthesizes these long chain ω-alkyl, β-hydroxy fatty acids have been identified, the gene encoding the β-hydroxyacyl-acyl carrier protein (ACP) dehydratase activity has remained elusive. Recent bioinformatics-based studies and drug inhibition experiments have identified the M. tuberculosis gene Rv0636 as a promising candidate for this role. Using a recently described, specialized transduction-based genetic tool we now demonstrate that MSMEG1341, the Mycobacterium smegmatis homologue of Rv0636, is an essential gene; null mutants of the gene could only be generated in a merodiploid strain which contained a second integrated acetamide-inducible copy of MSMEG1341. Growth of the conditional mutant in the absence of acetamide resulted in loss of mycolic acid biosynthesis and eventually loss of viability due to cell lysis. Null MSMEG1341 mutants could also be generated in a M. smegmatis strain containing an integrated copy of Rv0636, indicating that Rv0636 was the functional counterpart of MSMEG1341 in M. tuberculosis. Our results demonstrate that MSMEG1341 is an essential gene involved in mycolic acid biosynthesis and encodes the FAS-II β-hydroxyacyl-ACP dehydratase.

INTRODUCTION

Tuberculosis affects a third of the world population and causes 1.8 million fatalities annually (Dye, 2006). The spread of TB has been facilitated in recent years due to the susceptibility of HIV-infected individuals to Mycobacterium tuberculosis, the aetiological agent of TB (Paolo & Nosanchuk, 2004). The problem has also been compounded by the emergence of multi-drug-resistant TB (MDR-TB) (Kaye & Frieden, 1996) and extensively drug-resistant (XDR)-TB strains (Wright et al., 2007). The aetiological agent of TB (Paolo & Nosanchuk, 2004). The problem has also been compounded by the emergence of multi-drug-resistant TB (MDR-TB) (Kaye & Frieden, 1996) and extensively drug-resistant (XDR)-TB strains (Wright et al., 2007). The aetiological agent of TB (Paolo & Nosanchuk, 2004). The problem has also been compounded by the emergence of multi-drug-resistant TB (MDR-TB) (Kaye & Frieden, 1996) and extensively drug-resistant (XDR)-TB strains (Wright et al., 2007).

The biosynthesis of mycolic acids is linked to the unusual presence of two fatty acid synthases in mycobacteria: a multi-functional mammalian-type fatty acid synthase-I (FAS-I) (Smith et al., 2003), and a bacterial-type multi-enzyme complex fatty acid synthase-II (FAS-II), in which dissociable enzymes interact with an acyl carrier protein (ACP), AcpM, that manoeuvres the growing fatty acyl chain between their active sites (Kremer et al., 2001). M. tuberculosis FAS-I conducts de novo synthesis of intermediate length (principally C16 and C24) fatty acids. On the other hand, FAS-II, while incapable of de novo fatty acid synthesis, extends FAS-I generated primers to long chain fatty acids (C48–C56), denoted as meromycolic acids.

In M. tuberculosis, the process is initiated by the condensation of acyl-CoA and malonyl-ACP, a reaction catalysed by mtFabH, a β-ketoacyl-ACP synthase (Brown et al., 2005). The newly formed β-ketoacyl-ACP is first reduced by a β-ketoacyl-ACP-reductase (MabA) (Banerjee et al., 2004) to form a β-hydroxyacyl-ACP intermediate which is then dehydrated by a yet unidentified β-hydroxyacyl-ACP-dehydratase to form an enoyl-ACP intermediate.
This is further reduced by an enoyl-ACP-reductase (InhA) to yield an ACP-bound acyl chain that is two carbons longer (Banerjee et al., 1994; Kikuchi & Kusaka, 1984). Subsequent reductive FAS-II cycles are initiated by two other β-ketoacyl-ACP synthases, KasA and KasB, (Krement al., 2000; Mdluli et al., 1998; Schaeffer et al., 2001) to form a meromeric acid. The mero-chain is then condensed with a C_{26} fatty acid (Gande et al., 2004; Portevin et al., 2005; Takayama et al., 2005) in a reaction catalysed by Pks13, to yield an oxo-meromyc acid intermediate which is then reduced to form a mature mycolic acid (Lea-Smith et al., 2007).

Genes encoding FAS-II enzymes are essential for mycobacterial survival (Bhatt et al., 2005; Parish et al., 2007; Vilcheze et al., 2000); conditional depletion or inactivation of these enzymes leads to bacterial cell lysis (Bhatt et al., 2005; Vilcheze et al., 2000). This makes FAS-II enzymes attractive targets for drug development. Indeed, two well-studied anti-tubercular agents, isoniazid and thiolaomycin, both target FAS-II enzymes (Banerjee et al., 1994; Kremer et al., 2000). While the genes for most FAS-II enzymes had been identified in two separate loci in the 1990s (Banerjee et al., 1994, 1998; Cole et al., 1998), the ORF encoding the β-hydroxyacyl-ACP-dehydratase has remained elusive (BLAST searches of E. coli dehydratases FabZ and FabA failed to identify a specific mycobacterial homologue). The first putative dehydratase candidates were identified using bioinformatics in a key study which demonstrated that seven M. tuberculosis proteins contained a double hot dog fold closely related to that of (R)-enoyl CoA-hydratase from Aeromonas caviae (PDB code 1IQ6) (Castell et al., 2005). Of these, two genes, Rv3538 and Rv6036, were proposed to be essential for mycobacterial growth (Sassetti et al., 2003), though only the latter has a homologue in Mycobacterium leprae which also synthesizes mycolic acids. Rv6036 shares 21% identity and 37% similarity with 1IQ6 over the whole protein (Brown et al., 2007), suggesting that Rv6036 was the most likely candidate for the FAS-II dehydratase activity. In a recent study we demonstrated that flavonoid-based inhibitors of dehydratases from Escherichia coli and Plasmidium falciparum were also active against Mycobacterium, bovis BCG (Brown et al., 2007). Growth of M. bovis BCG in the presence of these compounds caused cessation of mycolic acid biosynthesis and growth inhibition. Furthermore, expression of multiple copies of plasmid-borne Rv6036 in M. bovis BCG increased its resistance to the flavonoid compounds and restored the biosynthesis of mycolic acids, suggesting that the product of Rv6036 was the likely target of these dehydratase inhibitors. The compounds also caused inhibition of Mycobacterium smegmatis FAS-II in a whole-cell in vitro assay (Brown et al., 2007). The bioinformatics analysis and drug inhibition studies together suggested that the putative protein encoded by Rv6036 was likely the FAS-II dehydratase. In this study we chose MSMEG1341, the M. smegmatis homologue of Rv6036, to address the question whether Rv6036 and MSMEG1341 do indeed encode the mycobacterial FAS-II dehydratase. Utilizing CESTET (conditional expression specialized transcription essentiality test), a previously described method for testing gene essentiality and conditionally depleting essential gene products (Bhatt et al., 2005), we have shown that MSMEG1341 is an essential gene in M. smegmatis. Furthermore, the role of MSMEG1341 as the FAS-II β-hydroxyl-acyl-ACP dehydratase was probed by depleting the enzyme in a conditional mutant.

METHODS

**Bacterial strains, phages, plasmids.** All plasmids, phages and bacterial strains used in this study are outlined in Table 1. Strains of E. coli were cultured in LB broth. M. smegmatis strains were grown in Tryptic Soy Broth (TSB; Difco) containing 0.05% Tween-80 (TSBT). Solid media were made by adding 1.5% agar to the above-mentioned broths. The concentrations of antibiotics used were 100 μg ml⁻¹ for hygromycin and 20 μg ml⁻¹ for kanamycin with M. smegmatis and 150 μg ml⁻¹ for hygromycin and 40 μg ml⁻¹ for kanamycin with E. coli.

**Construction of merodiploid strains.** The E. coli–Mycobacterium inducible shuttle vector pSD26 containing the acetamidase promoter and encoding a 6-histidine C-terminal tag was used for the construction of pSD26 MSMEG1341 and pSD26 Rv6036, using M. smegmatis mc²155 and M. tuberculosis H37Rv DNA, respectively. All DNA manipulations were performed using standard protocols, as described by Sambrook & Russell (2001). PCR amplification was performed using the M. smegmatis primers 5′-GATCGATCGATCCATGTGAGTT-3′ and 5′-GATCGATCGATCGGTTGCGGCGC-3′ and M. tuberculosis primers 5′-GATCGATCGATCCATGGCCTGAGTT-3′ and 5′-GATCGATCGATCGGTTGCGGCGC-3′, which contain BamHI and EcoRV restriction sites, respectively (underlined). The 454 bp PCR product was then digested with BamHI and EcoRV and ligated with similarly digested pSD26, giving rise to pSD26 MSMEG1341 and pSD26 Rv6036. The coding sequence of the recombinant gene was verified by DNA sequencing. The single-copy-integrating constructs pABMSMEG1341 and pABRv6036 were constructed by ligating approximately 3.2 kb XbaI–ClaI fragments from the pSD26 MSMEG1341 and pSD26 Rv6036 constructs (containing the M. smegmatis MSMEG1341 and the M. tuberculosis Rv6036 genes cloned in-frame, downstream of the inducible M. smegmatis acetamidase promoter) into XbaI–ClaI-digested pVM306. The merodiploid strains mc²155::pABMSMEG1341 and mc²155::pABRv6036 were obtained by electroporation with mc²155 pABMSMEG1341 and pABRv6036, respectively, and selecting for kanamycin-resistant colonies (electroporation of M. smegmatis was performed as described earlier by Snapper et al., 1990).

**Construction of deletion mutants.** Approximately 1 kb sequences of the upstream and downstream regions of MSMEG1341 were PCR amplified from M. smegmatis mc²155 genomic DNA using the primer pairs MS1341LL (5′-TTTTTTTTTCTAAAGTTGCTCACTCCCAGAGGCCTTC-3′) and MS1341LR (5′-TTTTTTTTTCTAAAGTTGCTCACTCCCAGAGGCCTTC-3′), and MS1341RL (5′-TTTTTTTTTTCTAAAGTTGCTCACTCCCAGAGGCCTTC-3′), and MS1341LR (5′-TTTTTTTTTTCTAAAGTTGCTCACTCCCAGAGGCCTTC-3′), respectively. The PCR fragments were digested with Van91I (sites were incorporated in the primers) and cloned into Van91I-digested p9004S (gift from T. Hsu and W. R. Jacobs Jr, Albert Einstein College of Medicine, New York). The recombinant plasmids
Table 1. Plasmids, bacterial strains, and phages used in this study

<table>
<thead>
<tr>
<th>Plasmid, strain, or phage</th>
<th>Description</th>
<th>Reference/source</th>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pMV306</td>
<td>KanR, single-copy-integrating vector; inserts into the phage L5 chromosomal integrating site attB in many mycobacteria</td>
<td>Stover et al., 1991</td>
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<tr>
<td>pSD26</td>
<td>HygR, multiple copy, expression vector with the acetamidase promoter</td>
<td>Dauglet al., 2003</td>
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<td>pSD26-MSMEG1341</td>
<td>M. smegmatis mc²155 MSMEG1341 cloned into pSD26</td>
<td>This work</td>
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<tr>
<td>pSD26-Rv0636</td>
<td>M. tuberculosis H37Rv Rv0636 cloned into pSD26</td>
<td>This work</td>
</tr>
<tr>
<td>pABMSMEG1341</td>
<td>XbaI–ClaI fragment of pSD26-MSMEG1341 subcloned into pMV306</td>
<td>This work</td>
</tr>
<tr>
<td>pABBRv0636</td>
<td>XbaI–ClaI fragment of pSD26-Rv0636 subcloned into pMV306</td>
<td>This work</td>
</tr>
<tr>
<td>p0004s</td>
<td>Vector for cloning allelic-exchange substrates to be used for specialized transduction; contains φ phage cos site and HygR marker (hyg)</td>
<td>Gift from Tsungda Hsu and W. R. Jacobs Jr</td>
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<tr>
<td>pΔMSMEG1341</td>
<td>Derivative of p0004s designed for allelic exchange of M. smegmatis MSMEG1341</td>
<td>This work</td>
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<td><strong>Bacterial strains</strong></td>
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<tr>
<td>mc²155 : : pABMSMEG1341</td>
<td>Electroporation-proficient ept mutant of M. smegmatis strain mc²6</td>
<td>Snapper et al., 1990</td>
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<td>mc²155 : : pABRv0636</td>
<td>KanR mc²155 derivative containing pABMSMEG1341 integrated into the attB site</td>
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<td>ΔMSMEG1341-A</td>
<td>Native copy of MSMEG1341 in mc²155 : : pABMSMEG1341 is replaced by hyg</td>
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<tr>
<td>ΔMSMEG1341-B</td>
<td>Native copy of MSMEG1341 in mc¹55 : : pABRv0636 is replaced by hyg</td>
<td>This work</td>
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<td><strong>Phages</strong></td>
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<td>phAE159</td>
<td>Conditionally replicating shuttle phasmid derived from the lytic mycobacteriophage TM4</td>
<td>Bardarov et al., 2002</td>
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<tr>
<td>phΔMSMEG1341</td>
<td>Derivative of phAE159 obtained by cloning pΔMSMEG1341 into its unique PacI site</td>
<td>This work</td>
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*aΔB, Phage L5 chromosomal integration site.

obtained after transforming E. coli TOP-10 cells were sequenced to confirm that there were no errors in the PCR amplified sequences. One plasmid, pΔMSMEG1341, was digested with PacI and used for packaging into the temperature sensitive mycobacteriophage phAE159 as described previously (Bardarov et al., 2002) to yield phasmid DNA of the knockout phage phΔMSMEG1341. Generation of high titre phage particles and CESTET were performed as described earlier (Bardarov et al., 2002; Bhatt et al., 2005). PCR verification of allelic exchange was performed using the primer pair MS1341LL and et al., 2005). PCR verification of allelic exchange was performed using the primer pair MS1341LL and MS1341RR.

### Conditional depletion of MSMEG1341

The M. smegmatis strain ΔMSMEG1341-A and ΔMSMEG1341-B were grown in TSBT and 0.2 % acetamide to an OD₆₀₀ of 0.5. The cells were washed twice in TSBT to remove traces of acetamide and resuspended to the original volume in TSBT. This cell suspension was used as a 20 % inoculum in TSBT and grown for 12 h to deplete intracellular MSMEG1341/Rv0636. The depleted culture was then used to inoculate TSBT with or without 0.2 % acetamide (5 % inoculum). This inoculation point of the depleted culture corresponds to the time ‘0’ for all experiments designed to test cell density, viability and mycolate and lipid profiles.

### Determination of the in vivo effects of MSMEG1341 depletion on fatty acid and mycolic acid synthesis

Samples of ΔMSMEG1341-A grown in the presence or absence of acetamide were taken at time points 0, 6, 12, 24 h and labelled with 1 μCi ml⁻¹ (37 kBq ml⁻¹) [1,2-14C]acetate [57 μCi mmol⁻¹ (2.1 GBq mmol⁻¹), GE Healthcare, Amersham Biosciences], followed by incubation at 37°C for 3 h. The 14C-labelled cells were harvested by centrifugation at 2000 g followed by washing with PBS. The cell pellet was subjected to alkaline hydrolysis using 5 % aqueous tetrabutylammonium hydroxide (TBAH) at 100°C overnight, followed by the addition of 4 ml CH₂Cl₂, 500 μl CH₃I and 2 ml water, followed by mixing for 30 min. The upper aqueous phase was discarded following centrifugation and the lower organic phase washed thrice with water and evaporated to dryness. The resulting fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were dissolved in diethyl ether, insoluble residues were removed by centrifugation and the ether solution evaporated to dryness and redissolved in 200 μl CH₂Cl₂. Equivalent counts (25 000 c.p.m.) of the resulting solution of FAMEs and MAMEs were subjected to thin-layer chromatography (TLC) using silica gel plates (5735 silica gel 60F₂₅₄, Merck), developed in petroleum ether–acetone (95 : 5). Autoradiograms were produced by overnight exposure of Kodak X-Omat AR film to the plates to reveal 14C-labelled FAMEs and MAMEs.

### RESULTS

#### Bioinformatic analysis of Rv0636 and flanking genes

The putative peptide encoded by Rv0636 showed the presence of a hydratase-2 motif (G-D-X-N-P-L-I-V-H-X₅-A) found in the ‘lid’ region of hot dog fold-containing dehydratases (Castell et al., 2005), and included the catalytic residues [D-X₁-H] (Qin et al., 2000). Structure predictions based on A. caviae dehydratase crystal structure 1IQ6 revealed that Rv0636 has the hydratase-2 motif-containing ‘lid’ in a similar orientation as 1IQ6, E. coli FabA and P. falciparum FabZ, consistent with the hot dog fold postulated to be necessary for dehydratase activity (data not shown). A closer analysis of genomic sequences flanking Rv0636 indicated that the gene may be co-transcribed with Rv0635 and Rv0637: the former ORF

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overlaps with Rv0636 while the start codon of Rv0637 is only 3 bp downstream of the stop codon of Rv0636. Indeed, the operon prediction website of The Institute of Genomic Research, USA, lists the genes as part of a predicted operon (http://www.tigr.org/tigr-scripts/operons/operons.cgi). Interestingly, while neither flanking gene shows any similarity to Rv0636, the putative Rv0635-encoded peptide is 48% identical and 65% similar to that encoded by Rv0637. While Rv0635, like Rv0636, is an essential gene, Rv0637 is not essential for growth (Sassetti et al., 2003). These findings suggested that Rv0635, Rv0636 and Rv0637 may be functionally related, and that Rv0635 and Rv0637 may encode similar functions. Furthermore, these genes are situated close to the mmaA1-mmaA4 cluster involved in biosynthesis of oxygenated mycolic acids. MSMEG1341 is the M. smegmatis homologue of Rv0636 and both M. smegmatis and M. leprae show a similar arrangement of ORFs to those found in the Rv0635-Rv0637 region of M. tuberculosis (Fig. 1). We chose the M. smegmatis gene MSMEG1341 for further genetic analysis of this gene cluster.

**MSMEG1341 is essential in M. smegmatis**

Specialized transduction of M. smegmatis mc2^155 with phAMSMEG1341, a temperature-sensitive phage containing an allelic-exchange substrate designed to replace MSMEG1341 with a hygromycin resistance cassette (hyg), failed to yield any hygromycin-resistant (Hgy^R) transductants on TSB agar (TSBA) (data not shown), suggesting that MSMEG1341 was essential for the growth of M. smegmatis. CESTET (Bhatt et al., 2005) was used to confirm the essentiality of MSMEG1341. Firstly, a mehidiploid strain was generated by integration of pABMSMEG1341 into the M. smegmatis mc2^155 chromosome. The plasmid is a single copy integrative vector that contains MSMEG1341 cloned downstream of the acetamide-induced acamidase promoter (Mahenthiralingam et al., 1993). The resultant strain mc2^155::pABMSMEG1341, when transduced with phAMSMEG1341, yielded Hgy^R colonies only on TSBA plates containing acetamide. The replacement of the native chromosomal copy of MSMEG1341 in these transductants was confirmed by PCR (Fig. 2) and Southern blot (data not shown). One transductant was chosen for further analysis and was denoted ΔMSMEG1341-A. Subculture of ΔMSMEG1341-A on TSBA with or without acetamide showed that strain could grow only in the presence of acetamide (Fig. 3a), demonstrating that acamidase-promoter-driven expression of the non-native copy of MSMEG1341 in ΔMS1341-A was essential for its survival, thus confirming the essentiality of MSMEG1341.

**Conditional depletion of MSMEG1341 causes mycobacterial cell lysis**

Previous studies demonstrated that conditional depletion of two FAS-II enzymes, InhA and KasA, in CESTET-derived conditional M. smegmatis mutants resulted in cell lysis (Bhatt et al., 2005). If MSMEG1341 was the FAS-II dehydratase, loss of its activity would be expected to result in cell lysis. The growth of ΔMSMEG1341-A in liquid medium with or without acetamide was monitored over 24 h. While the strain grew normally in medium containing acetamide, the culture in the medium without acetamide showed a decrease in OD600 values with time (data not shown), resulting in a clearly lysed culture after 24 h of incubation (Fig. 3c). Monitoring of viable counts
demonstrated that the culture grown in the absence of acetamide showed a decrease in viability. After 10 h of growth the culture grown in the absence of acetamide had more than a log fewer colony forming units than that grown in the presence of acetamide (Fig. 3b). This result indicated that, like other FAS-II enzymes, depletion of MSMEG1341 was bactericidal and resulted in cell lysis, making it likely that MSMEG1341 was the FAS-II dehydratase.

Fig. 3. (a) Growth of ΔMSMEG1341-A on TSB agar with or without acetamide. Growth of the strain is dependent on the acetamide inducible expression of the second recombinant copy of MSMEG1341, thus demonstrating the essentiality of MSMEG1341 for growth of M. smegmatis. (b) Plot of viable counts (c.f.u.) of ΔMSMEG1341-A grown in TSBT with (●) or without (○) acetamide. (c) Pictures of the cultures of ΔMSMEG1341-A grown for 24 h in TSBT with or without acetamide.

Loss of MSMEG1341 results in cessation of mycolic acid biosynthesis

Loss of FAS-II dehydratase activity would be expected to lead to a loss of mycolic acid biosynthesis. To assess the effects of depletion of MSMEG1341 in the conditional mutant, we pulsed cultures of ΔMS1341-A with [14C]acetate at different time intervals after inoculation into TSBT with or without acetamide. Total mycolic acids were extracted from the pulsed cultures as MAMEs and separated by TLC. While the culture grown in the presence of acetamide showed no alterations in the levels of newly synthesized mycolates, a decrease was observed in the culture grown in the absence of acetamide within 6 h of growth (Fig. 4). The decrease in the latter was not accompanied by a decrease in the level of labelled FAS-I-synthesized fatty acids up to 12 h (Fig. 4). These results clearly demonstrated the role of MSMEG1341 in mycolic acid biosynthesis. In addition, the above phenotype of MAMEs isolated either from chloroform/methanol lipid extracts or from delipidated cells (i.e. cell wall-bound MAMEs) afforded similar results (data not shown).

Rv0636 is the functional equivalent of MSMEG1341 in M. tuberculosis

While sequence comparisons and the corresponding organization of flanking genes in M. tuberculosis and M. smegmatis indicated that Rv0636 and MSMEG1341 were homologues, it was necessary to confirm that the products of both genes were functionally equivalent. To this end, we introduced Rv0636 into M. smegmatis mc2155 by single copy integration of the plasmid pABRv0636. Similar to pABMSMEG1341, the expression of Rv0636 could be controlled by the acetamidase promoter. The resulting strain mc2155::pABRv0636 was then transduced with phΔMSMEG1341. A HygR transductant obtained on an acetamide-containing plate was analysed by PCR and was found to contain a deletion of MSMEG1341, replacing it...
with hyg (data not shown). Furthermore, the strain (denoted ΔMS1341-B), was unable to grow on a plate lacking acetamide on subsequent subculture (Fig. 5a), and growth in liquid medium devoid of acetamide led to cell lysis (Fig. 5b). These data demonstrated that Rv0636 could functionally complement the putative M. smegmatis FAS-II dehydratase and therefore be denoted as the M. tuberculosis FAS-II dehydratase.

**DISCUSSION**

When the genes encoding KasA/B, MabA and InhA, the core enzymes of the FAS-II reductive cycle, were first identified in two different regions of the M. tuberculosis chromosome (Banerjee et al., 1998; Cole et al., 1998), no ORF encoding a dehydratase was detected in either locus. Following the identification of an R-specific hydratase hot dog fold in M. tuberculosis Rv0636 (Castell et al., 2005), we demonstrated that overexpression of Rv0636 in mycobacteria conferred resistance to known dehydratase inhibitors (Brown et al., 2007). Thus, the data available from bioinformatics-based studies and drug inhibition experiments collectively suggested that Rv0636 was the FAS-II β-hydroxyacyl-ACP-dehydratase required for mycolic acid biosynthesis. Subsequently, we selected the M. smegmatis gene MSMEG1341, the homologue of M. tuberculosis Rv0636, for generating a mutant strain. Using the conditional mutant ΔMSMEG1341 we demonstrated that M. smegmatis MSMEG1341 was an essential gene and that, like other FAS-II enzymes (Bhatt et al., 2005; Vilcheze et al., 2000), depletion of MSMEG1341 led to loss of viability and caused cell lysis. Loss of MSMEG1341 was also shown to correlate with a cessation of mycolic acid biosynthesis. Together, the molecular modelling data, essentiality of MSMEG1341 and the loss of mycolic acid biosynthesis and subsequent lysis following depletion in a conditional MSMEG1341 mutant established that MSMEG1341 encoded the FAS-II dehydratase component in M. smegmatis. Furthermore, null MSMEG1341 mutants could also be generated in an M. smegmatis strain containing an inducible copy of M. tuberculosis Rv0636, confirming that Rv0636 was the functional homologue of MSMEG1341 and was thus the M. tuberculosis FAS-II dehydratase.

Due to the emergence of MDR-TB (Kaye & Frieden, 1996), and more recently XDR-TB (Wright et al., 2006), it has become imperative to look for new drug targets. Due to the essential nature of mycolic acids in mycobacterial virulence and survival, enzymes involved in the biosynthesis of these unique fatty acids have always been attractive targets for drug development (Schroeder et al., 2002). Isoniazid and ethionamide target InhA and derivatives of thiolactomycin have been tested against KasA (Banerjee et al., 1994; Kremer et al., 2000). With the identification of the dehydratase, an additional component of FAS-II can now be targeted for development of drugs that inhibit mycolic acid biosynthesis. Indeed, as mentioned above, certain flavonoid-derived compounds have already been tested and found to inhibit FAS-II activity (Brown et al., 2007). Our identification of M. tuberculosis Rv0636 and M. smegmatis MSMEG1341 as the FAS-II dehydratase completes the identification of all the genes encoding the core FAS-II enzymes and opens up the possibility of testing compounds known to target dehydratases in other bacteria. The development of an in vitro assay for Rv0636 activity would greatly facilitate these studies.

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**REFERENCES**


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