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 α -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.

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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.*, 2006). *M. tuberculosis* and other mycobacteria have a distinct cell wall which has a lipid-rich outer layer that is highly impermeable (Minnikin, 1982). One of the major components of this outer envelope are mycolic acids, long chain α -alkyl, β -hydroxy fatty acids that are essential for bacterial survival (Bhatt *et al.*, 2005; Parish *et al.*, 2007; Portevin *et al.*, 2004; Vilcheze *et al.*, 2000). These are either found esterified to the non-reducing termini of an arabinogalactan-peptidoglycan cell wall core, or are present in the

free glycolipid trehalose dimycolate (TDM) (Brennan & Nikaido, 1995; Dover *et al.*, 2004).

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.*, 1998) 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.

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Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; DTT, dithiothreitol; FAS, fatty acid synthase; FAMES, fatty acid methyl esters; MAMES, mycolic acid methyl esters.

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, (Kremer *et al.*, 2000; Mdluli *et al.*, 1998; Schaeffer *et al.*, 2001) to form a meromycolic 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-mycolic 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 thiolactomycin, 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 *Rv0636*, were proposed to be essential for mycobacterial growth (Sasseti *et al.*, 2003), though only the latter has a homologue in *Mycobacterium leprae* which also synthesizes mycolic acids. *Rv0636* shares 21% identity and 37% similarity with 1IQ6 over the whole protein (Brown *et al.*, 2007), suggesting that *Rv0636* 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 *Plasmodium 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 *Rv0636* in *M. bovis* BCG increased its resistance to the flavonoid compounds and restored the biosynthesis of mycolic acids, suggesting that the product of *Rv0636* 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 *Rv0636* was likely the FAS-II dehydratase. In this study we chose *MSMEG1341*, the *M. smegmatis* homologue of *Rv0636*, to address the question whether *Rv0636* and

MSMEG1341 do indeed encode the mycobacterial FAS-II dehydratase. Utilizing CESTET (conditional expression specialized transduction 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 $\mu\text{g ml}^{-1}$ for hygromycin and 20 $\mu\text{g ml}^{-1}$ for kanamycin with *M. smegmatis* and 150 $\mu\text{g ml}^{-1}$ for hygromycin and 40 $\mu\text{g ml}^{-1}$ 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-*Rv0636*, 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'-GATCGATCGGATCCATGGCTCTGCGTGAGTT-3' and 5'-GATCGATCGATATCGGCGAGTCGTGCGGTGGCGA-3' and *M. tuberculosis* primers 5'-GATCGATCGGATCCATGGCGTGCGTGAGTT-3' and 5'-GATCGATCGATATCCGCTAACTTCGCCGA-3', which contain *Bam*HI and *Eco*RV restriction sites, respectively (underlined). The 454 bp PCR product was then digested with *Bam*HI and *Eco*RV and ligated with similarly digested pSD26, giving rise to pSD26-*MSMEG1341* and pSD26-*Rv0636*. The coding sequence of the recombinant gene was verified by DNA sequencing. The single-copy-integrating constructs pABMSMEG1341 and pABRv0636 were constructed by ligating approximately 3.2 kb *Xba*I-*Cl*aI fragments from the pSD26-*MSMEG1341* and pSD26-*Rv0636* constructs (containing the *M. smegmatis* *MSMEG1341* and the *M. tuberculosis* *Rv0636* genes cloned in-frame, downstream of the inducible *M. smegmatis* acetamidase promoter) into *Xba*I-*Cl*aI-digested pMV306. The merodiploid strains mc²155::pABMSMEG1341 and mc²155::pABRv0636 were obtained by electroporating mc²155 with pABMSMEG1341 and pABRv0636, 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'-TTTTTTTCCATAAATTGGCTCAGTCGGCAGAGCGTTTC-3') and MS1341LR (5'-TTTTTTTCCATTCTTGGTCCCAGTGGATCGGGTTGAG-3'), and MS1341RL (5'-TTT-TTTTCCATAGATTGGTTCAACGCCGGGTCAAGTC-3') and MS1341RR (5'-TTTTTTTCCATCTTTTGGTCGTCGCTCACCGCATCTC-3'), respectively. The PCR fragments were digested with *Van*9II (sites were incorporated in the primers) and cloned into *Van*9II-digested p0004S (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

Plasmid, strain, or phage	Description	Reference/source
Plasmids		
pMV306	Kan ^R , single-copy-integrating vector; inserts into the phage L5 chromosomal integrating site <i>att_B</i> * in many mycobacteria	Stover <i>et al.</i> , 1991
pSD26	Hyg ^R , multiple copy, expression vector with the acetamidase promoter	Daugelat <i>et al.</i> , 2003
pSD26-MSMEG1341	<i>M. smegmatis</i> mc ² 155 MSMEG1341 cloned into pSD26	This work
pSD26-Rv0636	<i>M. tuberculosis</i> H37Rv Rv0636 cloned into pSD26	This work
pABMSMEG1341	<i>Xba</i> I– <i>Cl</i> aI fragment of pSD26-MSMEG1341 subcloned into pMV306	This work
pABRv0636	<i>Xba</i> I– <i>Cl</i> aI fragment of pSD26-Rv0636 subcloned into pMV306	This work
p0004s	Vector for cloning allelic-exchange substrates to be used for specialized transduction; contains λ phage <i>cos</i> site and Hyg ^R marker (<i>hyg</i>)	Gift from Tsungda Hsu and W. R. Jacobs Jr
p Δ MSMEG1341	Derivative of p0004s designed for allelic exchange of <i>M. smegmatis</i> MSMEG1341	This work
Bacterial strains		
mc ² 155	Electroporation-proficient <i>ept</i> mutant of <i>M. smegmatis</i> strain mc ² 6	Snapper <i>et al.</i> , 1990
mc ² 155::pABMSMEG1341	Kan ^R mc ² 155 derivative containing pABMSMEG1341 integrated into the <i>att_B</i> site	This work
mc ² 155::pABRv0636	Kan ^R mc ² 155 derivative containing pABRv0636 integrated into the <i>att_B</i> site	This work
Δ MSMEG1341-A	Native copy of MSMEG1341 in mc ² 155::pABMSMEG1341 is replaced by <i>hyg</i>	This work
Δ MSMEG1341-B	Native copy of MSMEG1341 in mc ² 155::pABRv0636 is replaced by <i>hyg</i>	This work
Phages		
phAE159	Conditionally replicating shuttle phasmid derived from the lytic mycobacteriophage TM4	Bardarov <i>et al.</i> , 2002
ph Δ MSMEG1341	Derivative of phAE159 obtained by cloning p Δ MSMEG1341 into its unique <i>Pac</i> I site	This work

**att_B*, 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, p Δ MSMEG1341, was digested with *Pac*I 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 ph Δ MSMEG1341. 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 MS1341RR.

Conditional depletion of MSMEG1341. The *M. smegmatis* strains Δ MSMEG1341-A and Δ MSMEG1341-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 Δ MSMEG1341-A grown in the presence or absence of acetamide were taken at time points 0, 6, 12, 24 h and labelled with 1 μ Ci ml⁻¹ (37 kBq ml⁻¹) [1,2-¹⁴C]acetate [57 mCi mmol⁻¹ (2.1 GBq mmol⁻¹), GE Healthcare, Amersham Bioscience], 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 μ l 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 μ l 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-X₄-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

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 (Sasseti *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* mc²155 with phΔ*MSMEG1341*, 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 (Hyg^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 mero-diploid strain was generated by integration of pAB*MSMEG1341* into the *M. smegmatis* mc²155 chromosome. The plasmid is a single copy integrative vector that contains *MSMEG1341* cloned downstream of the acetamide-induced acetamidase promoter (Mahenthiralingam *et al.*, 1993). The resultant strain mc²155::pAB*MSMEG1341*, when transduced with phΔ*MSMEG1341*, yielded Hyg^R colonies only on TSBA plates containing acetamide. The replacement of the native chromosomal copy of *MSMEG1341* in these transductants was con-

Fig. 1. Maps of the genomic regions of *M. tuberculosis* *Rv0636*, *M. smegmatis* mc²155 *MSMEG1341* and *M. leprae* TN *ML1909*. The inverted triangles represent tRNA-encoding genes.

Fig. 2. (a) Map of the *MSMEG1341* region in the *M. smegmatis* mc²155::pAB*MSMEG1341* genome and its corresponding region in the conditional mutant Δ*MSMEG1341-A*. *res*, γδ resolvase site; *hyg*, hygromycin resistance gene from *Streptomyces hygroscopicus*; *sacB*, sucrose counterselectable gene from *Bacillus subtilis*. (b) Agarose gel electrophoresis of PCR products obtained from genomic DNA isolated from mc²155 (lane 1) or Δ*MSMEG1341-A* (lane 2); M, DNA size marker. The primers used for PCR amplification are indicated by small arrows in the map and the expected sizes of the PCR products are indicated with straight lines.

firmed by PCR (Fig. 2) and Southern blot (data not shown). One transductant was chosen for further analysis and was denoted Δ*MSMEG1341-A*. Subculture of Δ*MSMEG1341-A* on TSBA with or without acetamide showed that strain could grow only in the presence of acetamide (Fig. 3a), demonstrating that acetamidase-promoter-driven expression of the non-native copy of *MSMEG1341* in Δ*MS1341-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 Δ*MSMEG1341-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

Fig. 3. (a) Growth of Δ MSMEG1341-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 Δ MSMEG1341-A grown in TSBT with (●) or without (○) acetamide. (c) Pictures of the cultures of Δ MSMEG1341-A grown for 24 h in TSBT with or without acetamide.

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.

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 Δ MS1341-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 ϕ ΔMSMEG1341. A Hyg^R transductant obtained on an acetamide-containing plate was analysed by PCR and was found to contain a deletion of MSMEG1341, replacing it

Fig. 4. Autoradiograph (Kodak X-Omat AR film) exposed to TLC plate used for separating ¹⁴C-labelled methyl esters of mycolic acids (MAMEs) extracted from cultures of Δ MSMEG1341-A grown in TSBT with or without acetamide (extraction and TLC protocols are described in Methods). α , α' and epoxy correspond to α -mycolates, α' -mycolates and epoxy mycolates respectively. FAMES, fatty acid methyl esters.

with *hyg* (data not shown). Furthermore, the strain (denoted Δ MSMEG1341-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 by synergy 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 Δ MSMEG1341 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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Fig. 5. (a) Growth of MSMEG1341 knockout generated in a strain expressing an acetamide-inducible copy of *M. tuberculosis* Rv0636 (Δ MSMEG1341-B) on TSB agar plates with or without acetamide. (b) Cultures of the same strain in TSBT with or without acetamide after 24 h of incubation.

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