Deletion of a mycobacterial gene encoding a reductase leads to an altered cell wall containing β-oxo-mycolic acid analogues, and the accumulation of long-chain ketones related to mycolic acids


School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K.

*These authors contributed equally

† Correspondence:

Tel: +44-(0)121-4158123, Fax: +44-(0)121-4145925, E-mail: a.bhatt@bham.ac.uk

Running Title: Mycobacterial mycolic acid reductase
SUMMARY

Mycolic acids are essential components of the mycobacterial cell wall. In this study we show that a gene encoding a reductase involved in the final step of mycolic acid biosynthesis can be deleted in *Mycobacterium smegmatis* without affecting cell viability. Deletion of *MSMEG4722* (ortholog of *Mycobacterium tuberculosis Rv2509*) altered culture characteristics and antibiotic sensitivity. The ∆*MSMEG4722* strain synthesized α-alkyl, β-oxo intermediates of mycolic acids which were found esterified to cell wall-arabinogalactan. While the precursors could not be isolated directly due to their inherent instability during base-treatment, their presence was established by prior reduction of the β-oxo group by sodium borohydride. Interestingly, the mutant also accumulated unsaturated ketones, similar to tuberculenone from *M. tuberculosis*, which were shunt products derived from spontaneous decarboxylation of α-alkyl, β-oxo fatty acid precursors of mycolic acids.
INTRODUCTION

Mycolic acids are a major and essential component of the lipid-rich cell envelope of the human pathogen *Mycobacterium tuberculosis* and other related mycobacteria. Found either covalently attached to the terminal arabinose residues of the mycolyl arabinogalactan-peptidoglycan (mAGP) complex, or as the free glycolipids, trehalose monomycolate (TMM), trehalose dimycolate (TDM) and glucose monomycolate (GMM), these α-alkyl, β-hydroxyl long chain fatty acids play an important role in reduced cell wall permeability (Brennan and Nikaido, 1995; Daffe and Draper, 1998; Gao et al., 2003; Jackson et al., 1999) and virulence (Bhatt et al., 2007; Dubnau et al., 2000; Gao et al., 2003; Glickman et al., 2000; Rao et al., 2006) of mycobacteria.

In *M. tuberculosis*, a multifunctional Type-I fatty acid synthase (FAS-I) synthesizes C_{16-18} and C_{24-26} fatty acids in a bimodal fashion. The former is then channelled to a Type-II, multienzyme complex called fatty acid synthase II (FAS-II), which through its iterative reductive cycles extends the acyl chain to long chain meromycolic acids (C_{56-64}) (Bloch, 1975, 1977; Brindley et al., 1969; Peterson and Bloch, 1977). Finally, a polyketide synthase, Pks13 catalyzes the Claisen condensation of a C_{26} fatty acid and a mero-acid to yield an α-alkyl, β-oxo acyl intermediate which in turn is reduced to form a mature mycolic acid (Gande et al., 2004; Portevin et al., 2004) (Figure 1A). While earlier studies were focussed on identifying genes encoding ‘core’ FAS-II enzymes, not much was known about the final, post-Pks13 step of mycolic acid biosynthesis: the reduction of the β-oxo group to a hydroxyl group leading to the formation of the mycolic acid motif. Unlike in mycobacteria, genes encoding enzymes involved in the biosynthesis of mycolic acids are non-essential in corynebacteria, facilitating the generation of null mutants. Recently, Lea-Smith et al. (2007) generated a mutant of *Corynebacterium glutamicum NCgl2385* that had a slow growth phenotype, and produced corynomycolate precursors with a β-oxo group. In the
same study, the authors also used bioinformatics to identify Rv2509, the *M. tuberculosis*
ortholog of NCgl2385, as a possible candidate for reduction of the mycolic acid motif (Lea-
Smith et al., 2007). *Mycobacterium smegmatis* has often been used as a surrogate for *M.
tuberculosis* when studying biosynthetic pathways. The fast growing, non-pathogenic *M.
smegmatis* strain is particularly useful in studying cell wall biosynthesis genes since it can
tolerate deletion of some genes that are essential in *M. tuberculosis* (Amin et al., 2008; Escuyer
et al., 2001). Moreover, while the two species differ in mero-chain modifications, core enzymes
involved in mycolate biosynthesis are interchangeable (Brown et al., 2007; Parish et al., 2007).
We thus chose to address the role of Rv2509 in mycobacterial mycolic acid biosynthesis by
generating a deletion mutant of MSMEG4722, the *M. smegmatis* homologue of Rv2509.

RESULTS

*MSMEG4722* and *Rv2509* encode proteins structurally similar to short chain
reductases/dehydrogenases

Using bioinformatics, Lea-Smith et al. (2007) identified *M. tuberculosis* Rv2509 as the
homologue of NCgl2385, the *C. glutamicum* reductase involved in mycolic acid motif formation.
The closest match for Rv2509 in the *M. smegmatis* mc²155 genome was the putative protein
MSMEG4722 (Figure 1B). Both predicted proteins contained conserved active site residues and
residues for NAD/NADP binding (Figure 1B). Predictions of the three-dimensional structures of
proteins often give insights into potential catalytic properties. We used the @TOME server to
screen for known structures of proteins that were predicted to be most closely related to Rv2509
(Douguet and Labesse, 2001). Predictions of E-values from TITO and 3D-PSSM servers (-
121140 and 1.23e-02, respectively) strongly suggested that 1cyd (Mus musculus carbonyl reductase complexed with NADPH and 2-propanol) was the closest match to Rv2709 (22% sequence identity). Using the 1cyd co-ordinates and the FUGUE server (Shi et al., 2001) we generated an in silico 3D structure of Rv2509. The predictions revealed similar 3D structural folds for 1cyd, Rv2509 and the E. coli fatty acid reductase, FabG (data not shown). Additionally, when the NADPH moiety from 1cyd was superimposed in the predicted NADP-binding fold of Rv2509, the predicted distances between the conserved residues and the co-factor showed a fit similar to that seen in 1cyd (data not shown). These data suggested that Rv2509 was likely a NAD/NADP-dependent mycobacterial reductase. As outlined above, the homologous M. smegmatis gene MSMG4722 was chosen for further analysis.

Deletion of MSMG4722 in M. smegmatis mc²155 alters culture characteristics and sensitivity to antibiotics

To study the role of MSMG4722 in mycolic acid motif formation, we deleted MSMG4722 in M. smegmatis mc²155 by specialized transduction (Bardarov et al., 2002) (Figure 2A). The ability to generate a null mutant indicated that MSMG4722 was not essential for the viability of M. smegmatis mc²155. Loss of MSMG4722 had a remarkable effect of the colony morphology of M. smegmatis mc²155 on TSB agar. While the colonies of the parental, wild type strain mc²155 were glossy, those of the mutant strain ΔMSMG4722 appeared to have a dry surface (Figure 2B). The change was more apparent when the strains were grown on TSB agar supplemented with Tween-80. Unlike colonies of the parental strain mc²155 which had a smooth surface, colonies of ΔMSMG4722 had an irregular, convoluted surface (Figure 2B).
The ΔMSMEG4722 mutant also showed a slightly slower growth rate than the parental mc²155 strain (Figure 2C; the OD₆₀₀ values at 24h correspond to 2×10⁸ and 10⁷ colony forming units/ml for mc²155 and ΔMSMEG4722 respectively). This wasn’t surprising as a similar growth defect was observed when the homologous gene was deleted in C. glutamicum, and a genome-wide transposon screen predicted that the loss of the homologous gene in M. tuberculosis would result in a slow growth phenotype (Lea-Smith et al., 2007; Sassetti et al., 2003). Additionally, when grown in LB broth, the mutant showed an increased sensitivity to the lipophilic antibiotic rifampicin (MIC=0.125 µg/ml) as compared to the parental strain mc²155 (MIC=16 µg/ml), but not to hydrophilic antibiotics like isoniazid and ethambutol. Wild type characteristics were restored on complementation of the ΔMSMEG4722 mutant with plasmid-borne MSMEG4722 indicating that the observed phenotypes in the mutant strain were solely due to the loss of MSMEG4722 (Figure 2B and C).

The ΔMSMEG4722 mutant failed to synthesize mature mycolic acids

The predicted role of MSMEG4722 in mycolic acid motif formation and the observed changes in the colony morphology of the ΔMSMEG4722 mutant prompted us to examine mycolic acids in the mutant strain. If MSMEG4722 was indeed the reductase catalyzing the conversion of the post Pks13, α-alkyl, β-oxo fatty acyl intermediate, then the ΔMSMEG4722 mutant would be expected to accumulate this unreduced intermediate of mycolic acid biosynthesis (Figure 1A). A standard procedure for release of mycolic acids from mycobacteria involves base hydrolysis of cells using tetrabutyl ammonium hydroxide (TBAH). This is followed by phase-transfer catalyzed derivatisation using methyl iodide that results in the formation of mycolic acid methyl
esters (MAMEs) (Dobson et al., 1985) which are analysed by TLC. Following base-treatment and derivatisation, [¹⁴C]-labelled extracts obtained from strains were analyzed by TLC. While α, α’ and epoxy MAMEs were present in the parental mc²155 strain, all three species were missing in the extract from the ΔMSMEG4722 strain (Figure 3A). Instead, the mutant strain showed the accumulation of a product(s) with a higher retardation factor (Rf) migrating above the methyl esters of fatty acids (FAMEs). When extracts from the mutant strain were analysed by 2D-Ag⁺-argentation TLC, the rapidly migrating species resolved into multiple subspecies in the second, Ag⁺-containing dimension indicating the presence of multiple species differing in degrees of unsaturation (Figure 3B). An identical result was obtained for extracts from delipidated cells which only contain cell-wall bound mycolates (Figure 3A) indicating that the observed changes in mycolate profiles applied to both total and specifically cell wall bound mycolates.

Mycolic acid biosynthesis was restored in the mutant strain following complementation with not only MSMEG4722, but also Rv2509, indicating that Rv2509 was a functional homologue of MSMEG4722 in M. tuberculosis (strains ΔMSMEG4722-C and ΔMSMEG4722-CRv respectively, Figure 3A).

The ΔMSMEG4722 mutant accumulates precursors of mycolic acids in the cell wall

The complete absence of α, α’ and epoxy MAMEs and appearance of new, rapidly migrating species in extracts of the ΔMSMEG4722 mutant suggested that these new species may either be precursors of mycolates or decomposition products of precursors generated as a result of the extraction procedure. The latter seemed more likely as base treatment of an un-reduced α-alkyl,
β-oxo fatty acid precursor, containing two oxo groups in close proximity has been shown to result in the generation of a palmitone-like decomposition product in corynebacteria (Walker et al., 1973). Such decomposition products would be expected to migrate similarly to the rapidly migrating species that were observed in the extracts of the mutant strain. To confirm whether unreduced precursors of α, α′ and epoxy mycolic acids were present in the mutant strain, cells were pre-treated with sodium borohydride (NaBH₄). This pretreatment has little effect on normal mycolates but results in the reduction of the β-oxo group in putative α-alkyl, β-oxo fatty acyl intermediates resulting in the formation of α-alkyl, β-hydroxy products, viz. mature mycolic acids. Thus, if the α-alkyl, β-oxo fatty acid precursors of α, α′ and epoxy mycolic acids did exist in the mutant strain, pre-treatment with NaBH₄ would be expected to convert these precursors into α, α′ and epoxy mycolic acids and TLC analysis of TBAH-treated, methylated extracts from NaBH₄-pretreated ΔMSMEG4722 cells would show the presence of α, α′ and epoxy MAMEs due to the prior reduction of the β-oxo group. When extracts of NaBH₄-pretreated cells were analyzed by TLC, species migrating with the same $R_f$ values as α, α′ and epoxy MAMEs were observed (Figure 3C). However, additional MAMEs were also present in the extracts. Two closely migrating species had a very low $R_f$ value and were detected in total mycolates strains from all strains (MAME-I; Figure 3C), while another with a $R_f$ value slightly greater than α-MAMEs was seen only in extracts from the mutant strain (MAME-II; Figure 3C).

MAME-I corresponded in chromatographic migration to two hydroxylated artefacts, characterised previously in acid methanolysates of mycobacteria having epoxy mycolates (Minnikin et al., 1982). Similar hydroxylated artefacts would be expected by NaBH₄ reduction of the epoxy group in epoxy mycolates. Mass spectroscopic (MS) analysis confirmed these
findings with molecular sizes detected in all strains corresponding to those of α, α’, epoxy MAMEs and to the reduced, hydroxylated products of epoxy MAMEs (Table 2). Similar results were obtained when the analyses were performed on delipidated cells indicating that the α-alkyl, β-oxo fatty acyl precursors of mycolates from M. smegmatis were esterified to the AG in the cell wall (Figure 3C). However, in this case only a single MAME-I hydroxylated component was produced on NaBH₄-reduction of the delipidated cells (Figure 3C).

The NaBH₄-reduction of the β-oxo group, in a mycolate precursor, would result in two diastereoisomers of MAMEs which would migrate differently on TLC (Minnikin and Polgar, 1966). MAME-II, therefore, was likely to be a mycolate β-epimer and, on MALDI-TOF/MS, the molecular size was found to be identical to that of α-MAMES. Indeed, purified MAME-II co-migrated with a known standard for α-MAME β-epimer (data not shown). These results showed that the ΔMSMEG4722 mutant, due to loss of mycolyl reductase function, failed to make mature mycolic acids and instead synthesized the α-alkyl, β-oxo fatty acyl precursors of α, α’ and epoxy mycolates that were transported and subsequently esterified to the reducing termini of the AG-complex.

Lipid analysis revealed accumulation of a non-polar lipid in the ΔMSMEG4722 mutant

Polar and non-polar lipids labelled with [¹⁴C]-acetate were extracted from mc²155, ΔMSMEG4722 and ΔMSMEG4722-C strains and analyzed by 2D-TLC (Dobson et al., 1985). Interestingly, the lipids corresponding to TMM and TDM in mc²155 were replaced by two lipids with slightly altered mobilities in the mutant strain (Figure 4A). Both lipids had a slightly higher
$R_f$ value ($R_f$ 0.147 and $R_f$ 0.39) than parental TMM and TDM ($R_f$ 0.117 and $R_f$ 0.36) in direction 1. This was likely due to esterification of the $\alpha$-alkyl, $\beta$-oxo mycolic acid precursors rather than mature mycolic acids to trehalose in the mutant strain as was observed in the C. glutamicum mutant (Lea-Smith et al., 2007). Furthermore, the mutant strain showed accumulation of a non-polar species accompanied by a total loss of free mycolic acids (Figure 4B, C). This lipid species, referred to as Lipid-Y, did not stain with Dittmer-Lester reagent or with $\alpha$-napthol-sulfuric acid indicating the absence of phosphate groups and carbohydrates (data not shown). Following purification by preparative TLC, Lipid-Y was characterized by MALDI-TOF/MS, and NMR (Figure 5). Three species of $m/z$ 907.6, 935.6 and 963.6 were observed in the mass spectra (Figure 5A) indicating a difference of $m/z$ 28 between each species. Further, $^1$H-NMR and $^{13}$C-NMR revealed a signal characteristic of $-\text{CH}_2-$ groups (1.3 ppm and 30 ppm, respectively) and indicated the presence of alkyl chains differing ($\text{CH}_2)_2$ units. Additionally, $^1$H-NMR and $^{13}$C-NMR provided evidence for the presence of cis and/or trans double bonds (1, 2, 3, Figure 5B-D) with latter possessing an adjacent methyl branch, (5, Figure 5B-D) and a keto group (14, Figure 5C-D) suggesting that Lipid-Y was a mixture of cis and trans-unsaturated long chain ketones (Figure 5D). Based on the masses obtained by MALDI-TOF/MS, these unsaturated, branched-ketones were of chain lengths C$_{62}$, C$_{64}$ or C$_{66}$. Since the accumulation of Lipid-Y was accompanied by a loss of mycolates (and no detectable free $\alpha$-alkyl, $\beta$-oxo mycolate precursors), it seemed likely that the ketones that comprised Lipid-Y were derived from free $\beta$-oxo precursors. Free $\alpha$-alkyl, $\beta$-oxo mycolate precursors could undergo decarboxylation to form ketones. If this was the case, then the oxo group would be situated between alkyl chains that originate from a meroacid on one side and the $\alpha$-branch on the other. Using Electron Impact-Mass Spectroscopy (EI-MS) we were able to confirm the presence of a
fragment of m/z 351 corresponding to the α-chain (C_{22}) plus a carbonyl group. In addition, the detection of fragments of m/z 546, 574 and 602 corresponding to C_{39}, C_{41} and C_{43} mono-unsaturated alkyl chains respectively further substantiated our findings.

DISCUSSION

With the exception of KasB, all enzymes involved in the biosynthesis of mycolic acids in mycobacterial species are encoded by essential genes (Bhatt et al., 2005; Brown et al., 2007; Parish et al., 2007; Portevin et al., 2004; Sacco et al., 2007). However, we were able to generate a viable null mutant of MSMEG4722, the gene that encodes the reductase involved in mycolic acid motif synthesis in M. smegmatis. This was not entirely surprising because global transposon mutagenesis screens predicted insertions in Rv2509, the M. tuberculosis homologue of MSMEG4722, to result in a slow growth phenotype. Indeed, the ΔMSMEG4722 mutant did exhibit a slow growth rate similar to what was observed in C. glutamicum (Lea-Smith et al., 2007).

Alkaline hydrolysis of the parental strain M. smegmatis mc^{3}155 released α-, α’- and epoxy mycolates, as expected, but hydrolysates of the ΔMSMEG4722 mutant had no evidence for mycolates (Figure 3A). Instead, hydrolysates of the mutant ΔMSMEG4722 showed the presence of rapidly migrating components (labelled ‘?’, Figure 3A, B). If the ΔMSMEG4722 mutant was accumulating α-alkyl, β-oxo mycolate precursors, alkaline hydrolysis would produce unstable β-oxo acids, which would lose carbon dioxide to yield long-chain ketones. Using an
alternative approach, we confirmed the presence of the $\alpha$-alkyl, $\beta$-oxo fatty acyl precursors by converting them to $\alpha$-alkyl, $\beta$-hydroxy fatty acids (mycolates) by prior reduction of bound mycolates, using NaBH$_4$. This resulted in the appearance of $\alpha$, $\alpha'$ and epoxy-MAMEs in extracts of both whole and delipidated cells from the mutant strain (Figure 3C).

As expected (Minnikin and Polgar, 1966), reduction of the $\beta$-oxo mycolate precursors gave a mixture of separable diastereoisomers and the presence of the $\beta$-epimer of $\alpha$-MAME was clearly seen (MAME-II, Figure 3C). It would be expected that the $\beta$-epimers of $\alpha'$- and epoxy mycolates would also be produced, but such minor compounds would not be readily seen on 1D-TLC (Figure 3C). The epoxy function is also susceptible to NaBH$_4$ reduction and isomeric hydroxylated derivatives were identified (MAME-I, Figure 3C), corresponding to two artefacts previously characterised in acid methanolysates (Minnikin et al., 1982). However, reduction of the delipidated cells gave only the most polar hydroxylated derivative (Figure 3C). This strongly suggests that access of NaBH$_4$ to cell wall bound epoxy mycolates was restricted. It has recently been shown that keto mycolates from *Mycobacterium bovis* BCG adopt a folded “W” conformation (Villeneuve et al., 2007) with the keto group in a similar hydrophilic environment as the hydroxy acid unit. It is reasonable to suggest that bound epoxy mycolic acids might also prefer such a folded “W” conformation that could direct the access of NaBH$_4$ in a regiospecific manner, resulting in the formation of only a single hydroxylated derivative. It is notable that the covalently bound $\beta$-oxo precursors in the $\Delta$MSMEG4722 mutant also produce only the more polar derivative. This would suggest that $\beta$-oxo precursors also fold in the same way as intact mycolates, indicating that a $\beta$-hydroxy group is not absolutely essential prerequisite for folding in a “W” conformation. Indeed, the fact that $\Delta$MSMEG4722 mutant cells are viable, with $\beta$-oxo
mycolate analogues in their envelopes, strongly suggests that an exchange of a native $\beta$-hydroxy
group for an unnatural $\beta$-oxo unit is permitted. However, as discussed later, the $\Delta$MSMEG4722
mutant cells were more permeable to lipophilic antibiotics and colony morphology was affected.
Further studies will be needed to clarify these intriguing observations.

Additionally, replacement of TMM and TDM with derivatives with slightly altered TLC
mobility in $\Delta$MSMEG4722 (Figure 4A) suggested that the $\alpha$-alkyl, $\beta$-oxo mycolate precursors
were also esterified to trehalose. Indeed, we obtained similar results for MAME analysis from
extracts of whole cells (which contain wall bound and trehalose bound mycolates) as well as
delipidated cells (which contain only wall bound mycolates) demonstrating that mycolic acids in
both the mAGP complex and in TMM/TDM were replaced by the $\alpha$-alkyl, $\beta$-oxo fatty acid
precursors.

While the loss of the reductase was expected to generate precursors of mycolic acids, it
was surprising that the $\alpha$-alkyl, $\beta$-oxo fatty acids were associated with the cell wall. These data
suggested that mycobacterial components involved in the processing, transport and subsequent
transfer of mycolic acids to the cell wall (including the hypothetical mycolyl transferases I and II
and the proteins of the Antigen 85 complex; [(Takayama et al., 2005)]) were probably able to do
the same with the $\alpha$-alkyl, $\beta$-oxo fatty acid intermediates. In contrast, Lea-Smith et al (Lea-
Smith et al., 2007) reported reduced levels of AG-mycolylation in the C. glutamicum reductase
mutant ($\Delta$NCgl2385). Corynomycolate derivatives released from mutant cell wall were distinct
from wild type corynomycolates and showed a $\sim$80% reduction in abundance. However, the
extraction method used in this previous study involved acid-methanolyis. In light of our findings
it is likely that rather than a reduction in mycolylation, the $\Delta$NCgl2385 mutant contained equally
abundant α-alkyl, β-oxo corynomycolate precursors esterified to the AG which were not
detected by GC and MS because of decomposition resulting from acid-methanolysis.

Incorporation of the α-alkyl, β-oxo fatty acid precursors, instead of mycolic acids in the
cell wall had a significant change in the characteristics of the cell wall of *M. smegmatis*
rendering the mutant strain more susceptible to lipophilic antibiotics due to an increased
permeability. The presence on a β-oxo rather than a β-hydroxyl group also affected the colony
morphology of the mutant strain, presumably due to changes in the hydrophobicity of the outer
surface of the bacterial cells.

A key distinction between the reductase mutants of *C. glutamicum* and *M. smegmatis* was
the accumulation of an unusual lipid, Lipid-Y, in the latter. MS and NMR analysis of purified
Lipid-Y revealed it to be a mixture of unsaturated, branched ketones. Similar ketones were
detected in strains of *Mycobacterium tuberculosis* (tuberculenone) and *Corynebacterium
diptheriae* (Asselineau, 1954; Pudles and Lederer, 1954). The total number of carbons (C\textsubscript{60±C\textsubscript{3}})
in the mono unsaturated *M. tuberculosis* ketone, tuberculenone is similar to those of Lipid-Y
(C\textsubscript{62}–C\textsubscript{66}) in the *MSMEG4722* mutant. However, tuberculenone was characterised before the
advent of mass spectrometry (Asselineau, 1954) so precise comparisons are not meaningful. It
has been suggested that ketones like tuberculenone are derived from decarboxylation of the α-
alcohol, β-oxo fatty acid intermediates of mycolic acids (Asselineau, 1966). In addition to cell wall
bound and glycolipid associated mycolates, mycobacteria also contain free mycolic acids and
would be expected to contain some transient, unreduced mycolic acid intermediates at any given
time. It is likely that tuberculenone is derived from these intermediates. In the Δ*MSMEG4722*
mutant however no free mycolic acids were detected (Figure 4C). Instead, an accumulation of
α-alkyl, β-oxo fatty acid intermediates would be expected to occur. Free acids of such intermediates would then undergo decarboxylation to form the ketones that comprise Lipid-Y. Using EI-MS we were able to confirm that this was indeed the case.

Environmental mycobacteria are known alter cell wall mycolate composition in response to growth substrates resulting in a more hydrophobic wall when grown in the presence of hydrophobic substrates (Wick et al., 2002). It is not clear whether mycolate reduction is regulated by environmental factors but our studies herein have shown that loss of the β-hydroxy mycolate-motif reduction alters cell wall hydrophobicity. Whether this has an effect in vivo (in the case of M. tuberculosis) remains to be studied.

In conclusion, our results clearly demonstrate that MSMEG4722 is the reductase involved in generation of the mycolic acid motif in M. smegmatis. The loss of this function is not lethal, allowing cell wall incorporation of β-oxo mycolate analogues but affecting the growth characteristics of the bacterium.

SIGNIFICANCE

Mycolic acid biosynthesis is essential for mycobacterial survival and many antituberculosis drugs like isoniazid, ethionamide and thiolactomycin target enzymes of this exclusive pathway (Banerjee et al., 1994; Kremer et al., 2000). Interestingly, MSMEG4722, which catalyses the final step in mycolic acid biosynthesis in M. smegmatis, is non-essential and α-alkyl, β-oxo mycolate precursors are attached to arabinogalactan and trehalose. However, we have demonstrated that loss of function does cause major changes in the cell wall of M. smegmatis,
making it more susceptible to lipophilic antibiotics, such as rifampicin. By extension, loss of
Rv2509, the *M. tuberculosis* homologue, would be expected to have a bearing not only on
susceptibility to antibiotics, but also on virulence as strains of *M. tuberculosis* with altered
mycolic acids are highly attenuated (Bhatt et al., 2007; Dubnau et al., 2000; Glickman et al.,
2000), highlighting the potential of Rv2509 as a ‘secondary’ drug target. Our studies also shed
some light on the post FAS-II/Pks13 processing, transport and transfer of mycolic acids to their
location in the cell envelope. The replacement of mycolic acids in the cell wall by the α-alkyl, β-
oxo fatty acid precursors suggested that post-Pks13 reduction of the β-oxo group was not
necessary for the subsequent processing pathways.

EXPERIMENTAL PROCEDURES

**Bacterial strains, phages, plasmids and culture conditions**

All plasmids, phages and bacterial strains used in this study are shown in Table 1. Strains of
*Escherichia coli* were cultured in Luria-Bertani Broth (LB; Difco). *M. smegmatis* strains were
grown in either LB broth or Tryptic Soy Broth (TSB; Difco), each containing 0.05 % Tween80.
TSB-agar was prepared by adding 1.5 % agar to TSB prior to autoclaving. For *M. smegmatis*
hygromycin (100 µg ml⁻¹) or kanamycin (20 µg ml⁻¹) was used for selection while hygromycin
(150 µg ml⁻¹) or kanamycin (40 µg ml⁻¹) was used for selecting recombinant *E. coli* strains.
Determination of minimum inhibitory concentrations (MIC) of antibiotics was done in LB Broth
using the Alamar Blue assay (Franzblau et al., 1998).
Bioinformatics

Sequence alignments were determined using BLAST or EBI ClustalW (Chenna et al., 2003) and rendered using the EScript 2.2 web server. Structural predictions were performed using the @TOME server (Douguet and Labesse, 2001) and modeling performed using the FUGUE web server (http://tardis.nibio.go.jp/fugue/align.php). PyMOL (DeLano Scientific) was used to create POV scenes followed by rendering by POV-Ray.

Construction of a MSMEG4722 null mutant

Approximately 1-kb sequences of the upstream and downstream regions of MSMEG4722 were PCR amplified from M. smegmatis mc²155 genomic DNA using the primer pairs MS4722LL (5’-TTTTTTTTCCATAAATTGGTGCCAGCAGTAGACG-3’) and MS4722LR (5’-TTTTTTTTCCATTTCTTGGAGTTCGGTGCAACGCTTC-3’), and MS4722RL (5’-TTTTTTTTCCATAGATTGGTGCCAGCAGTAGACG-3’) and MS4722RR (5’-TTTTTTTTCCATCTTTTGGAAACTGATCGCTCCAAGGG-3’) respectively (all primers had Van91I recognition sites incorporated at the 5’ end). The PCR fragments were digested with Van91I and directly cloned into Van91I digested p0004S (Gift from T. Hsu and W. R. Jacobs Jr., Albert Einstein College of Medicine, New York). Recombinant plasmids obtained after transforming E. coli TOP-10 cells were digested with Van91I for confirmation and sequenced. One plasmid, pΔMSMEG4722, was linearized by PacI digestion and packaged into the temperature sensitive mycobacteriophage phAE159 as described (Bardarov et al., 2002) to yield phasmid DNA of the knockout phage phΔMSMEG4722. Generation of high titre phage particles
and specialized transduction were performed as described earlier (Bardarov et al., 2002). Allelic
exchange in hygromycin-resistant transductants was confirmed by Southern blot.

Construction of complemented strains

*MSMEG4722* was PCR amplified from *M. smegmatis* mc²155 genomic DNA using the primers
MS4722-U (5'-GCAGGATCCAATGAGCCGATGCCAGTACCCG-3') and MS4722-D (5'-GCAGAATTCTAACCAGCCCGAGCTTCTTG-3') and cloned into the *E. coli-
Mycobacterium* shuttle plasmid pMV261 using the primer incorporated BamHI and EcoRI sites
to yield the recombinant plasmid pMV261-MSMEG4722. In a similar fashion, the plasmid
pMV261-Rv2509 was constructed using Rv2509 which was PCR amplified from *M. tuberculosis*
H37Rv genomic DNA using the primers Rv2509-U (5'-GCAGGATCCAATGCCGATACCCGCGCCC-3') and Rv2509-D (5'-GCAGAATTCTAAGCTGGCCCCCAAGCCTC-3'). The complemented strains  ΔMSMEG4722-
C and  ΔMSMEG4722-CRv were obtained by selecting kanamycin-resistant transformants
following electroporation of the mutant strain  ΔMSMEG4722 with pMV261-MSMEG4722 or
pMV261-Rv2509 respectively. Electroporation was done as described earlier (Snapper et al.,
1990).

Lipid and mycolic acid extraction and analysis

Polar and apolar lipids were extracted from *M. smegmatis* strains and analyzed as described
earlier (Dobson et al., 1985). For extraction of mycolic acid methyl esters (MAMEs), both the
delipidated cells and the whole cell pellets were subjected to alkaline hydrolysis using 5% aqueous tetrabutylammonium hydroxide (TBAH) at 100°C overnight, followed by the addition of 4 ml of CH₂Cl₂, 500 µl of CH₃I, 2 ml of 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 MAMEs were dissolved in diethyl ether, insoluble residues were removed by centrifugation and the ether solution evaporated to dryness and re-dissolved in 200 µl of CH₂Cl₂. Equivalent volumes of the resulting solution of FAMEs and MAMEs was subjected to thin-layer chromatography (TLC) using silica gel plates (5735 silica gel 60F₂₅₄; Merck, Darmstadt, Germany), 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. Argentation-TLC was performed as above after saturation of TLC plates with 10% aqueous silver nitrate solution and prior activation at 100°C for 1 h. Lipid-Y was purified by preparative silica gel TLC, using petroleum ether:ethyl acetate (98:2, v/v) and detection by spraying with ethanolic Rhodamine 6G to visualize the lipid under a 366 nm U.V. lamp. The area containing Lipid-Y was removed and extracted from the silica gel, using diethyl ether. The extracted sample was then resolved on a second TLC plate in toluene:acetone (95:5, v/v) and purified as above. Matrix-Assisted Laser Desorption Ionisation-Time of Flight/Mass Spectroscopy (MALDI-TOF/MS) of all samples was done using the Voyager DE-STR MALDI-TOF instrument (PerSeptive Biosystems, Framingham, MA). Nuclear Magnetic Resonance (NMR) spectra for Lipid-Y were recorded in CDCl₃ on a Bruker DRX500 operating at 500.13 MHz for $^1$H-NMR and 125.77 MHz for $^{13}$C-NMR.
ACKNOWLEDGEMENTS

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Gande, R., Gibson, K.J., Brown, A.K., Krumbach, K., Dover, L.G., Sahm, H., Shioyama, S., Oikawa, T., Besra, G.S., and Eggeling, L. (2004). Acyl-CoA carboxylases (accD2 and accD3), together with a unique polyketide synthase (Cg-pks), are key to mycolic acid biosynthesis in Corynebacterianeae such as Corynebacterium glutamicum and Mycobacterium tuberculosis. The J. Biol. Chem. 279, 44847-44857.


**FIGURE LEGENDS**

**Figure 1** Mycolic acid reductases in mycobacteria

(A) Schematic representation of the post-FAS-II steps in mycobacterial mycolic acid biosynthesis. AccD4 and AccD5 are acyl-CoA carboxylases while FadD32 is an acyl-AMP ligase. It is yet unclear whether the reduction of the $\beta$-oxo group occurs while the mycolic acid precursor is still attached to Pks13 or after release from Pks13 by a thioesterase. (B) Alignment of amino acid sequences of Rv2509 and MSMEG4722 with 1cyd (PDB file for the structure of *Mus musculus* carbonyl reductase with NADP and 2-propanol). $\alpha$-helices and $\beta$-sheets are indicated above the residues as coils and arrows respectively. Residues essential for NAD/NADP binding are indicated by triangles while the active site residue is indicated with a star.

**Figure 2** Generation of a *MSMEG4722* null mutant

(A) Map of the *MSMEG4722* region in the parental *M. smegmatis* strain mc²155 and its corresponding region in the ΔMSMEG4722 mutant. *res*, $\gamma\delta$ resolvase site; *hyg*, hygromycin resistance gene from *Streptomyces hygroscopicus*; *sacB*, sucrose counterselectable gene from *Bacillus subtilis*. Digoxigenin-labelled probes were derived from ~1kb upstream and downstream flanking sequences that were used to construct the knockout plasmid, and are indicated by thick lines with square ends. *ClaI* digested bands expected in a Southern blot are indicated in roman numerals with sizes in brackets. The inset shows the Southern blot of *ClaI* digested genomic DNA from the two strains with expected bands indicated by arrows. (B)
Colonies of wild type (mc²155), mutant (ΔMSMEG4722) and complemented (ΔMSMEG4722-C) strains on TSB-agar (-) or TSB-agar + 0.05% Tween-80 (+). Colony growth shown in upper row was obtained by inoculating 5µL of a broth culture on the agar plate while lower row shows pictures of a single isolated colony of each strain. Scale bar =1mm. (C) Growth curve of wild type (mc²155), mutant (ΔMSMEG4722) and complemented (ΔMSMEG4722-C) strains in TSB.

Figure 3  TLC analysis of mycolic acid methyl esters (MAMEs)

(A) TLC analysis of MAMEs extracted from mc²155, ΔMSMEG4722, ΔMSMEG4722-C and ΔMSMEG4722-Crv strains. The rapidly migrating species observed in ΔMSMEG4722 are indicated by a question mark. FAMEs; fatty acid methyl esters. (B) 2D Ag⁺-TLC of MAMEs extracted from mc²155, ΔMSMEG4722, ΔMSMEG4722-C. UFAMEs, unsaturated fatty acid methyl esters. (C) TLC analysis of MAMEs extracted from cells pre-treated with NaBH₄.

Figure 4  2D-TLC analysis of apolar lipids extracted from mc²155, ΔMSMEG4722 and ΔMSMEG4722-C strains

Panel (A), direction 1, chloroform:methanol:water, 100:14:0.8 (v/v); direction 2, chloroform:acetone:methanol:water, 50:60:2.5:3 (v/v). Panel (B), direction 1, petroleum ether:acetone, 98:2 (v/v, thrice); direction 2, toluene:acetone, 98:2 (v/v). Panel (C), direction 1, chloroform:methanol, 96:4 (v/v); direction 2, toluene:acetone, 80:20 (v/v). TMM, trehalose monomycolate; TDM, trehalose dimycolate; GMM, glucose monomycolate; FA, fatty acids; MA, mycolic acids. Lipid-Y is indicated by an arrow and question mark in Panel (B).
Figure 5 Structural analysis of Lipid-Y

(A) MALDI-TOF/MS (B) $^1$H-NMR and (C) $^{13}$C-NMR of purified Lipid-Y. Characteristic shifts are labelled as numbers and the structures they represent are indicated in (D).
A

B

C

Log10 OD600

Time (hours)
<table>
<thead>
<tr>
<th><strong>Table 1</strong></th>
<th><strong>Plasmids, bacterial strains, and phages used in this study</strong></th>
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</thead>
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<tr>
<td><strong>Plasmids</strong></td>
<td>Description</td>
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<tr>
<td>pMV261</td>
<td><em>E. coli</em>-Mycobacterium shuttle plasmid vector with <em>hsp60</em> promoter and Kan&lt;sup&gt;R&lt;/sup&gt;cassette (<em>aph</em>)</td>
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<tr>
<td>pMV261-MSMEG4722</td>
<td><em>MSMEG4722</em> cloned in pMV261</td>
</tr>
<tr>
<td>pMV261-Rv2509</td>
<td><em>Rv2509</em> cloned in pMV261</td>
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<tr>
<td>p0004s</td>
<td>Vector for cloning allelic-exchange substrates to be used for specialized transduction; contains λ phage cos site and Hyg&lt;sup&gt;R&lt;/sup&gt;cassette (<em>hyg</em>)</td>
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<tr>
<td>pΔMSMEG4722</td>
<td>Derivative of p0004s designed for allelic exchange of <em>M. smegmatis MSMEG4722</em></td>
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<td><strong>Bacterial strains</strong></td>
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<tr>
<td>mc&lt;sup&gt;2&lt;/sup&gt;155</td>
<td>Electroporation-proficient <em>ept</em> mutant of <em>M. smegmatis</em> strain mc&lt;sup&gt;2&lt;/sup&gt;6</td>
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<tr>
<td>ΔMSMEG4722</td>
<td>Deletion mutant of mc&lt;sup&gt;2&lt;/sup&gt;155 in which <em>MSMEG4722</em> is replaced by <em>hyg</em></td>
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<tr>
<td>ΔMSMEG4722-C</td>
<td>Complemented strain of ΔMSMEG4722 containing pMV261-MSMEG4722</td>
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<tr>
<td>ΔMSMEG4722-CRv</td>
<td>Complemented strain of ΔMSMEG4722 containing pMV261-Rv2509</td>
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<td><strong>Phages</strong></td>
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<tr>
<td>phAE159</td>
<td>Conditionally replicating shuttle phasmid derived from the lytic mycobacteriophage TM4</td>
</tr>
<tr>
<td>phΔMSMEG4722</td>
<td>Derivative of phAE159 obtained by cloning pΔMSMEG4722 into its unique <em>PacI</em> site</td>
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Table 2  MALDI-TOF/MS analysis of MAMEs isolated from different *M. smegmatis* strains. The masses indicated are those of Na-adducts and those of predominant species are shown in bold. α-epi is the isomer of α-mycolates.

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