Loss of a mycobacterial gene encoding a reductase leads to an altered cell wall containing beta-oxo-mycolic acid analogs and accumulation of ketones.

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1 **Deletion of a mycobacterial gene encoding a reductase leads to an altered cell**

2 **wall containing β-oxo-mycolic acid analogues, and the accumulation of long-**

3 **chain ketones related to mycolic acids**

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12 Running Title: Mycobacterial mycolic acid reductase

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1   **SUMMARY**

2   Mycolic acids are essential components of the mycobacterial cell wall. In this study we show

3   that a gene encoding a reductase involved in the final step of mycolic acid biosynthesis can be

4   deleted in *Mycobacterium smegmatis* without affecting cell viability. Deletion of *MSMEG4722*

5   (ortholog of *Mycobacterium tuberculosis Rv2509*) altered culture characteristics and antibiotic

6   sensitivity. The Δ*MSMEG4722* strain synthesized α-alkyl, β-oxo intermediates of mycolic acids

7   which were found esterified to cell wall-arabinogalactan. While the precursors could not be

8   isolated directly due to their inherent instability during base-treatment, their presence was

9   established by prior reduction of the β-oxo group by sodium borohydride. Interestingly, the

10 mutant also accumulated unsaturated ketones, similar to tuberculenone from *M. tuberculosis*,

11 which were shunt products derived from spontaneous decarboxylation of α-alkyl, β-oxo fatty

12 acid precursors of mycolic acids.

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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* mc[2]155 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 (-

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1 121140 and 1.23e-02, respectively) strongly suggested that 1cyd (*Mus musculus* carbonyl

2 reductase complexed with NADPH and 2-propanol) was the closest match to Rv2709 (22%

3 sequence identity). Using the 1cyd co-ordinates and the FUGUE server (Shi et al., 2001) we

4 generated an *in silico* 3D structure of Rv2509. The predictions revealed similar 3D structural

5 folds for 1cyd, Rv2509 and the *E. coli* fatty acid reductase, FabG (data not shown).

6 Additionally, when the NADPH moiety from 1cyd was superimposed in the predicted NADP-

7 binding fold of Rv2509, the predicted distances between the conserved residues and the co-

8 factor showed a fit similar to that seen in 1cyd (data not shown). These data suggested that

9 Rv2509 was likely a NAD/NADP-dependent mycobacterial reductase. As outlined above, the

10 homologous *M. smegmatis* gene *MSMEG4722* was chosen for further analysis.

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12 **Deletion of *MSMEG4722* in *M. smegmatis* mc^{2}155 alters culture characteristics and**

13 **sensitivity to antibiotics**

14 To study the role of *MSMEG4722* in mycolic acid motif formation, we deleted *MSMEG4722* in

15 *M. smegmatis* mc^{2}155 by specialized transduction (Bardarov et al., 2002) (Figure 2A). The

16 ability to generate a null mutant indicated that *MSMEG4722* was not essential for the viability of

17 *M. smegmatis* mc^{2}155. Loss of *MSMEG4722* had a remarkable effect of the colony morphology

18 of *M. smegmatis* mc^{2}155 on TSB agar. While the colonies of the parental, wild type strain

19 mc^{2}155 were glossy, those of the mutant strain Δ*MSMEG4722* appeared to have a dry surface

20 (Figure 2B). The change was more apparent when the strains were grown on TSB agar

21 supplemented with Tween-80. Unlike colonies of the parental strain mc^{2}155 which had a

22 smooth surface, colonies of Δ*MSMEG4722* had an irregular, convoluted surface (Figure 2B).

1   The Δ*MSMEG4722* mutant also showed a slightly slower growth rate than the parental mc^{2}155

2   strain (Figure 2C; the OD$_{600}$ values at 24h correspond to 2×10^8 and 10^7 colony forming units/ ml

3   for mc^{2}155 and Δ*MSMEG4722* respectively). This wasn't surprising as a similar growth defect

4   was observed when the homologous gene was deleted in *C. glutamicum*, and a genome-wide

5   transposon screen predicted that the loss of the homologous gene in *M. tuberculosis* would result

6   in a slow growth phenotype (Lea-Smith et al., 2007; Sassetti et al., 2003). Additionally, when

7   grown in LB broth, the mutant showed an increased sensitivity to the lipophilic antibiotic

8   rifampicin (MIC=0.125 μg/ml) as compared to the parental strain mc^{2}155 (MIC=16 μg/ml), but

9   not to hydrophilic antibiotics like isoniazid and ethambutol. Wild type characteristics were

10   restored on complementation of the Δ*MSMEG4722* mutant with plasmid-borne *MSMEG4722*

11   indicating that the observed phenotypes in the mutant strain were solely due to the loss of

12   *MSMEG4722* (Figure 2B and C).

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The Δ*MSMEG4722* mutant failed to synthesize mature mycolic acids

15   The predicted role of *MSMEG4722* in mycolic acid motif formation and the observed changes in

16   the colony morphology of the Δ*MSMEG4722* mutant prompted us to examine mycolic acids in

17   the mutant strain. If MSMEG4722 was indeed the reductase catalyzing the conversion of the

18   post Pks13, α-alkyl, β-oxo fatty acyl intermediate, then the Δ*MSMEG4722* mutant would be

19   expected to accumulate this unreduced intermediate of mycolic acid biosynthesis (Figure 1A). A

20   standard procedure for release of mycolic acids from mycobacteria involves base hydrolysis of

21   cells using tetrabutyl ammonium hydroxide (TBAH). This is followed by phase-transfer

22   catalyzed derivatisation using methyl iodide that results in the formation of mycolic acid methyl

1   esters (MAMEs) (Dobson et al., 1985) which are analysed by TLC. Following base-treatment

2   and derivatisation, [^{14}C]- labelled extracts obtained from strains were analyzed by TLC. While

3   α, α′ and epoxy MAMEs were present in the parental mc^{2}155 strain, all three species were

4   missing in the extract from the ΔMSMEG4722 strain (Figure 3A). Instead, the mutant strain

5   showed the accumulation of a product(s) with a higher retardation factor (R_f) migrating above the

6   methyl esters of fatty acids (FAMEs). When extracts from the mutant strain were analysed by

7   2D-Ag$^+$-argentation TLC, the rapidly migrating species resolved into multiple subspecies in the

8   second, Ag$^+$-containing dimension indicating the presence of multiple species differing in

9   degrees of unsaturation (Figure 3B). An identical result was obtained for extracts from

10 delipidated cells which only contain cell-wall bound mycolates (Figure 3A) indicating that the

11 observed changes in mycolate profiles applied to both total and specifically cell wall bound

12 mycolates.

13   Mycolic acid biosynthesis was restored in the mutant strain following complementation

14 with not only MSMEG4722, but also Rv2509, indicating that Rv2509 was a functional

15 homologue of MSMEG4722 in M. tuberculosis (strains ΔMSMEG4722-C and ΔMSMEG4722-

16 CRv respectively, Figure 3A).

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

19 The complete absence of α, α′ and epoxy MAMEs and appearance of new, rapidly migrating

20 species in extracts of the ΔMSMEG4722 mutant suggested that these new species may either be

21 precursors of mycolates or decomposition products of precursors generated as a result of the

22 extraction procedure. The latter seemed more likely as base treatment of an unreduced α-alkyl,

1   β-oxo fatty acid precursor, containing two oxo groups in close proximity has been shown to

2   result in the generation of a palmitone-like decomposition product in corynebacteria (Walker et

3   al., 1973). Such decomposition products would be expected to migrate similarly to the rapidly

4   migrating species that were observed in the extracts of the mutant strain. To confirm whether

5   unreduced precursors of α, α′ and epoxy mycolic acids were present in the mutant strain, cells

6   were pre-treated with sodium borohydride ($NaBH_4$). This pretreatment has little effect on

7   normal mycolates but results in the reduction of the β-oxo group in putative α-alkyl, β-oxo fatty

8   acyl intermediates resulting in the formation of α-alkyl, β-hydroxy products, *viz.* mature mycolic

9   acids. Thus, if the α-alkyl, β-oxo fatty acid precursors of α, α′ and epoxy mycolic acids did

10   exist in the mutant strain, pre-treatment with $NaBH_4$ would be expected to convert these

11   precursors into α, α′ and epoxy mycolic acids and TLC analysis of TBAH-treated, methylated

12   extracts from $NaBH_4$-pretreated Δ*MSMEG4722* cells would show the presence of α, α′ and

13   epoxy MAMEs due to the prior reduction of the β-oxo group. When extracts of $NaBH_4$ -

14   pretreated cells were analyzed by TLC, species migrating with the same R_f values as α, α′ and

15   epoxy MAMEs were observed (Figure 3C). However, additional MAMEs were also present in

16   the extracts. Two closely migrating species had a very low R_f value and were detected in total

17   mycolates strains from all strains (MAME-I; Figure 3C), while another with a R_f value slightly

18   greater than α-MAMEs was seen only in extracts from the mutant strain (MAME-II; Figure 3C).

19   MAME-I corresponded in chromatographic migration to two hydroxylated artefacts,

20   characterised previously in acid methanolysates of mycobacteria having epoxy mycolates

21   (Minnikin et al., 1982). Similar hydroxylated artefacts would be expected by $NaBH_4$ reduction

22   of the epoxy group in epoxy mycolates. Mass spectroscopic (MS) analysis confirmed these

1   findings with molecular sizes detected in all strains corresponding to those of α, α′, epoxy

2   MAMEs and to the reduced, hydroxylated products of epoxy MAMEs (Table 2). Similar results

3   were obtained when the analyses were performed on delipidated cells indicating that the α-alkyl,

4   β-oxo fatty acyl precursors of mycolates from *M. smegmatis* were esterified to the AG in the cell

5   wall (Figure 3C). However, in this case only a single MAME-I hydroxylated component was

6   produced on NaBH$_4$-reduction of the delipidated cells (Figure 3C).

7   The NaBH$_4$-reduction of the β-oxo group, in a mycolate precursor, would result in two

8   diastereoisomers of MAMEs which would migrate differently on TLC (Minnikin and Polgar,

9   1966). MAME-II, therefore, was likely to be a mycolate β-epimer and, on MALDI-TOF/MS,

10   the molecular size was found to be identical to that of α-MAMES. Indeed, purified MAME-II

11   co-migrated with a known standard for α-MAME β-epimer (data not shown). These results

12   showed that the Δ*MSMEG4722* mutant, due to loss of mycolyl reductase function, failed to make

13   mature mycolic acids and instead synthesized the α-alkyl, β-oxo fatty acyl precursors of α, α′

14   and epoxy mycolates that were transported and subsequently esterified to the reducing termini of

15   the AG-complex.

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17   **Lipid analysis revealed accumulation of a non-polar lipid in the Δ*MSMEG4722* mutant**

18   Polar and non-polar lipids labelled with [^{14}C]-acetate were extracted from mc^{2}155,

19   Δ*MSMEG4722* and Δ*MSMEG4722*-C strains and analyzed by 2D-TLC (Dobson et al., 1985).

20   Interestingly, the lipids corresponding to TMM and TDM in mc^{2}155 were replaced by two lipids

21   with slightly altered mobilities in the mutant strain (Figure 4A). Both lipids had a slightly higher

1   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

2   direction 1. This was likely due to esterification of the α-alkyl, β-oxo mycolic acid precursors

3   rather than mature mycolic acids to trehalose in the mutant strain as was observed in the *C.*

4   *glutamicum* mutant (Lea-Smith et al., 2007). Furthermore, the mutant strain showed

5   accumulation of a non-polar species accompanied by a total loss of free mycolic acids (Figure

6   4B, C). This lipid species, referred to as Lipid-Y, did not stain with Dittmer-Lester reagent or

7   with α-napthol-sulfuric acid indicating the absence of phosphate groups and carbohydrates (data

8   not shown). Following purification by preparative TLC, Lipid-Y was characterized by MALDI-

9   TOF/MS, and NMR (Figure 5). Three species of *m/z* 907.6, 935.6 and 963.6 were observed in

10   the mass spectra (Figure 5A) indicating a difference of *m/z* 28 between each species. Further,

11   ^{1}H-NMR and ^{13}C-NMR revealed a signal characteristic of -CH_2- groups (1.3 ppm and 30 ppm,

12   respectively) and indicated the presence of alkyl chains differing $(CH_2)_2$ units. Additionally, [1]H-

13   NMR and ^{13}C-NMR provided evidence for the presence of *cis* and/or *trans* double bonds (1, 2, 3

14   , Figure 5B-D) with latter possessing an adjacent methyl branch, (5, Figure 5B-D) and a keto

15   group (14, Figure 5C-D) suggesting that Lipid-Y was a mixture of *cis* and *trans*-unsaturated long

16   chain ketones (Figure 5D).   Based on the masses obtained by MALDI-TOF/MS, these

17   unsaturated, branched-ketones were of chain lengths C_{62}, C_{64} or C_{66}. Since the accumulation of

18   Lipid-Y was accompanied by a loss of mycolates (and no detectable free α-alkyl, β-oxo

19   mycolate precursors), it seemed likely that the ketones that comprised Lipid-Y were derived

20   from free β-oxo precursors.   Free α-alkyl, β-oxo mycolate precursors could undergo

21   decarboxylation to form ketones. If this was the case, then the oxo group would be situated

22   between alkyl chains that originate from a meroacid on one side and the α-branch on the other.

23   Using Electron Impact-Mass Spectroscopy (EI-MS) we were able to confirm the presence of a

1   fragment of *m/z* 351 corresponding to the α-chain (C_{22}) plus a carbonyl group. In addition, the

2   detection of fragments of *m/z* 546, 574 and 602 corresponding to C_{39}, C_{41} and C_{43} mono-

3   unsaturated alkyl chains respectively further substantiated our findings.

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DISCUSSION

7   With the exception of KasB, all enzymes involved in the biosynthesis of mycolic acids in

8   mycobacterial species are encoded by essential genes (Bhatt et al., 2005; Brown et al., 2007;

9   Parish et al., 2007; Portevin et al., 2004; Sacco et al., 2007). However, we were able to generate

10   a viable null mutant of *MSMEG4722*, the gene that encodes the reductase involved in mycolic

11   acid motif synthesis in *M. smegmatis*. This was not entirely surprising because global transposon

12   mutagenesis screens predicted insertions in *Rv2509*, the *M. tuberculosis* homologue of

13   *MSMEG4722*, to result in a slow growth phenotype. Indeed, the Δ*MSMEG4722* mutant did

14   exhibit a slow growth rate similar to what was observed in *C. glutamicum* (Lea-Smith et al.,

15   2007).

16   Alkaline hydrolysis of the parental strain *M. smegmatis* mc[2]155 released α-, α′- and

17   epoxy mycolates, as expected, but hydrolysates of the Δ*MSMEG4722* mutant had no evidence

18   for mycolates (Figure 3A). Instead, hydrolysates of the mutant Δ*MSMEG4722* showed the

19   presence of rapidly migrating components (labelled '?', Figure 3A, B). If the Δ*MSMEG4722*

20   mutant was accumulating α-alkyl, β-oxo mycolate precursors, alkaline hydrolysis would produce

21   unstable β-oxo acids, which would lose carbon dioxide to yield long-chain ketones. Using an

1   alternative approach, we confirmed the presence of the α-alkyl, β-oxo fatty acyl precursors by

2   converting them to α-alkyl, β-hydroxy fatty acids (mycolates) by prior reduction of bound

3   mycolates, using $NaBH_4$.   This resulted in the appearance of α, α´ and epoxy-MAMEs in

4   extracts of both whole and delipidated cells from the mutant strain (Figure 3C).

5   As expected (Minnikin and Polgar, 1966), reduction of the β-oxo mycolate precursors

6   gave a mixture of separable diastereoisomers and the presence of the β-epimer of α-MAME was

7   clearly seen (MAME-II, Figure 3C). It would be expected that the β-epimers of α′- and epoxy

8   mycolates would also be produced, but such minor compounds would not be readily seen on 1D-

9   TLC (Figure 3C). The epoxy function is also susceptible to $NaBH_4$ reduction and isomeric

10   hydroxylated derivatives were identified (MAME-I, Figure 3C), corresponding to two artefacts

11   previously characterised in acid methanolysates (Minnikin et al., 1982). However, reduction of

12   the delipidated cells gave only the most polar hydroxylated derivative (Figure 3C). This strongly

13   suggests that access of $NaBH_4$ to cell wall bound epoxy mycolates was restricted. It has recently

14   been shown that keto mycolates from *Mycobacterium bovis* BCG adopt a folded "W"

15   conformation (Villeneuve et al., 2007) with the keto group in a similar hydrophilic environment

16   as the hydroxy acid unit. It is reasonable to suggest that bound epoxy mycolic acids might also

17   prefer such a folded "W" conformation that could direct the access of $NaBH_4$ in a regiospecific

18   manner, resulting in the formation of only a single hydroxylated derivative. It is notable that the

19   covalently bound β-oxo precursors in the Δ*MSMEG4722* mutant also produce only the more

20   polar derivative. This would suggest that β-oxo precursors also fold in the same way as intact

21   mycolates, indicating that a β-hydroxy group is not absolutely essential prerequisite for folding

22   in a "W" conformation. Indeed, the fact that Δ*MSMEG4722* mutant cells are viable, with β-oxo

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1   mycolate analogues in their envelopes, strongly suggests that an exchange of a native β-hydroxy

2   group for an unnatural β-oxo unit is permitted. However, as discussed later, the Δ*MSMEG4722*

3   mutant cells were more permeable to lipophilic antibiotics and colony morphology was affected.

4   Further studies will be needed to clarify these intriguing observations.

5   Additionally, replacement of TMM and TDM with derivatives with slightly altered TLC

6   mobility in Δ*MSMEG4722* (Figure 4A) suggested that the α-alkyl, β-oxo mycolate precursors

7   were also esterified to trehalose. Indeed, we obtained similar results for MAME analysis from

8   extracts of whole cells (which contain wall bound and trehalose bound mycolates) as well as

9   delipidated cells (which contain only wall bound mycolates) demonstrating that mycolic acids in

10   both the mAGP complex and in TMM/TDM were replaced by the α-alkyl, β-oxo fatty acid

11   precursors.

12   While the loss of the reductase was expected to generate precursors of mycolic acids, it

13   was surprising that the α-alkyl, β-oxo fatty acids were associated with the cell wall. These data

14   suggested that mycobacterial components involved in the processing, transport and subsequent

15   transfer of mycolic acids to the cell wall (including the hypothetical mycolyl transferases I and II

16   and the proteins of the Antigen 85 complex; [(Takayama et al., 2005)]) were probably able to do

17   the same with the α-alkyl, β-oxo fatty acid intermediates. In contrast, Lea-Smith et al (Lea-

18   Smith et al., 2007) reported reduced levels of AG-mycolylation in the *C. glutamicum* reductase

19   mutant (Δ*NCgl2385*). Corynomycolate derivatives released from mutant cell wall were distinct

20   from wild type corynomycolates and showed a ~80% reduction in abundance. However, the

21   extraction method used in this previous study involved acid-methanolyis. In light of our findings

22   it is likely that rather than a reduction in mycolylation, the Δ*NCgl2385* mutant contained equally

1 abundant α-alkyl, β-oxo corynomycolate precursors esterified to the AG which were not

2 detected by GC and MS because of decomposition resulting from acid-methanolysis.

3   Incorporation of the α-alkyl, β-oxo fatty acid precursors, instead of mycolic acids in the

4 cell wall had a significant change in the characteristics of the cell wall of *M. smegmatis*

5 rendering the mutant strain more susceptible to lipophilic antibiotics due to an increased

6 permeability. The presence on a β-oxo rather than a β-hydroxyl group also affected the colony

7 morphology of the mutant strain, presumably due to changes in the hydrophobicity of the outer

8 surface of the bacterial cells.

9   A key distinction between the reductase mutants of *C. glutamicum* and *M. smegmatis* was

10 the accumulation of an unusual lipid, Lipid-Y, in the latter. MS and NMR analysis of purified

11 Lipid-Y revealed it to be a mixture of unsaturated, branched ketones. Similar ketones were

12 detected in strains of *Mycobacterium tuberculosis* (tuberculenone) and *Corynebacterium*

13 *diptheriae* (Asselineau, 1954; Pudles and Lederer, 1954). The total number of carbons ($C_{60} \pm C_3$)

14 in the mono unsaturated *M. tuberculosis* ketone, tuberculenone is similar to those of Lipid-Y

15 (C_{62}-C_{66}) in the *MSMEG4722* mutant. However, tuberculenone was characterised before the

16 advent of mass spectrometry (Asselineau, 1954) so precise comparisons are not meaningful. It

17 has been suggested that ketones like tuberculenone are derived from decarboxylation of the α-

18 alkyl, β-oxo fatty acid intermediates of mycolic acids (Asselineau, 1966). In addition to cell wall

19 bound and glycolipid associated mycolates, mycobacteria also contain free mycolic acids and

20 would be expected to contain some transient, unreduced mycolic acid intermediates at any given

21 time. It is likely that tuberculenone is derived from these intermediates. In the Δ*MSMEG4722*

22 mutant however no free mycolic acids were detected (Figure 4C). Instead, an accumulation of

1   α-alkyl, β-oxo fatty acid intermediates would be expected to occur.   Free acids of such

2   intermediates would then undergo decarboxylation to form the ketones that comprise Lipid-Y.

3   Using EI-MS we were able to confirm that this was indeed the case.

4   Environmental mycobacteria are known alter cell wall mycolate composition in response

5   to growth substrates resulting in a more hydrophobic wall when grown in the presence of

6   hydrophobic substrates (Wick et al., 2002).   It is not clear whether mycolate reduction is

7   regulated by environmental factors but our studies herein have shown that loss of the β-hydroxy

8   mycolate-motif reduction alters cell wall hydrophobicity.   Whether this has an effect *in vivo* (in

9   the case of *M. tuberculosis*) remains to be studied.

10   In conclusion, our results clearly demonstrate that MSMEG4722 is the reductase

11   involved in generation of the mycolic acid motif in *M. smegmatis.* The loss of this function is not

12   lethal, allowing cell wall incorporation of β-oxo mycolate analogues but affecting the growth

13   characteristics of the bacterium.

14

15   **SIGNIFICANCE**

16   Mycolic acid biosynthesis is essential for mycobacterial survival and many antituberculosis

17   drugs like isoniazid, ethionamide and thiolactomycin target enzymes of this exclusive pathway

18   (Banerjee et al., 1994; Kremer et al., 2000).   Interestingly, MSMEG4722, which catalyses the

19   final step in mycolic acid biosynthesis in *M. smegmatis*, is non-essential and α-alkyl, β-oxo

20   mycolate precursors are attached to arabinogalactan and trehalose.   However, we have

21   demonstrated that loss of function does cause major changes in the cell wall of *M. smegmatis*,

1   making it more susceptible to lipophilic antibiotics, such as rifampicin. By extension, loss of

2   *Rv2509*, the *M. tuberculosis* homologue, would be expected to have a bearing not only on

3   susceptibility to antibiotics, but also on virulence as strains of *M. tuberculosis* with altered

4   mycolic acids are highly attenuated (Bhatt et al., 2007; Dubnau et al., 2000; Glickman et al.,

5   2000), highlighting the potential of Rv2509 as a 'secondary' drug target. Our studies also shed

6   some light on the post FAS-II/Pks13 processing, transport and transfer of mycolic acids to their

7   location in the cell envelope. The replacement of mycolic acids in the cell wall by the α-alkyl, β-

8   oxo fatty acid precursors suggested that post-Pks13 reduction of the β-oxo group was not

9   necessary for the subsequent processing pathways.

10

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12   **EXPERIMENTAL PROCEDURES**

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

14   All plasmids, phages and bacterial strains used in this study are shown in Table 1. Strains of

15   *Escherichia coli* were cultured in Luria-Bertani Broth (LB; Difco). *M. smegmatis* strains were

16   grown in either LB broth or Tryptic Soy Broth (TSB; Difco), each containing 0.05 % Tween80.

17   TSB-agar was prepared by adding 1.5 % agar to TSB prior to autoclaving. For *M. smegmatis*

18   hygromycin (100 μg ml^{-1}) or kanamycin (20 μg ml^{-1}) was used for selection while hygromycin

19   (150 μg ml^{-1}) or kanamycin (40 μg ml^{-1}) was used for selecting recombinant *E. coli* strains.

20   Determination of minimum inhibitory concentrations (MIC) of antibiotics was done in LB Broth

21   using the Alamar Blue assay (Franzblau et al., 1998).

1

Bioinformatics

3   Sequence alignments were determined using BLAST or EBI ClustalW (Chenna et al., 2003) and

4   rendered using the EScript 2.2 web server. Structural predictions were performed using the

5   @TOME server (Douguet and Labesse, 2001) and modeling performed using the FUGUE web

6   server (http://tardis.nibio.go.jp/fugue/align.php). PyMOL (DeLano Scientific) was used to create

7   POV scenes followed by rendering by POV-Ray.

8

Construction of a *MSMEG4722* null mutant

10   Approximately 1-kb sequences of the upstream and downstream regions of *MSMEG4722* were

11   PCR amplified from *M. smegmatis* mc^{2}155 genomic DNA using the primer pairs MS4722LL

12   (5'-TTTTTTTTCCATAAATTGGTGGCCAGCAGGT AGTAGACG-3') and MS4722LR (5'-

13   TTTTTTTTCCATTTCTTGGAGTTCGGTGGCCAACG CTTC-3'), and MS4722RL (5'-

14   TTTTTTTTCCATAGATTGGTGGATCGACACCGAGTAC AC-3') and MS4722RR (5'-

15   TTTTTTTTCCATCTTTTGGAAACTGATCCGCTCCAAGGG-3') respectively (all primers

16   had *Van*91I recognition sites incorporated at the 5' end). The PCR fragments were digested with

17   *Van* 91I and directly cloned into *Van*91I digested p0004S (Gift from T. Hsu and W. R. Jacobs

18   Jr., Albert Einstein College of Medicine, New York). Recombinant plasmids obtained after

19   transforming *E. coli* TOP-10 cells were digested with *Van*91I for confirmation and sequenced.

20   One plasmid, p∆*MSMEG4722*, was linearized by *Pac*I digestion and packaged into the

21   temperature sensitive mycobacteriophage phAE159 as described (Bardarov et al., 2002) to yield

22   phasmid DNA of the knockout phage ph∆*MSMEG4722*. Generation of high titre phage particles

1 and specialized transduction were performed as described earlier (Bardarov et al., 2002). Allelic

2 exchange in hygromycin-resistant transductants was confirmed by Southern blot.

3

Construction of complemented strains

5 *MSMEG4722* was PCR amplified from *M. smegmatis* mc^{2}155 genomic DNA using the primers

6 MS4722-U (5'-GCAGGATCCAATGAGCCGCATGCCAGTACCCG-3') and MS4722-D (5'-

7 GCAGAATTCCTAACCGCCGCCGAGCTTCTTG-3') and cloned into the *E. coli-*

8 *Mycobacterium* shuttle plasmid pMV261 using the primer incorporated *Bam*HI and *Eco*RI sites

9 to yield the recombinant plasmid pMV261-*MSMEG4722*. In a similar fashion, the plasmid

10 pMV261-*Rv2509* was constructed using *Rv2509* which was PCR amplified from *M. tuberculosis*

11 H37Rv genomic DNA using the primers Rv2509-U (5'-

12 GCAGGATCCAATGCCGATACCCGCGCCC-3') and Rv2509-D (5'-

13 GCAGAATTCCTAGCTGCCCCCAAGCCTC-3'). The complemented strains Δ*MSMEG4722*-

14 C and Δ*MSMEG4722*-CRv were obtained by selecting kanamycin-resistant transformants

15 following electroporation of the mutant strain Δ*MSMEG4722* with pMV261-*MSMEG4722* or

16 pMV261-*Rv2509* respectively. Electroporation was done as described earlier (Snapper et al.,

17 1990).

18

Lipid and mycolic acid extraction and analysis

20 Polar and apolar lipids were extracted from *M. smegmatis* strains and analyzed as described

21 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 CH_2Cl_2, 500 µl of CH_3I, 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 CH_2Cl_2. Equivalent volumes of the resulting solution of FAMEs and MAMEs was subjected to thin-layer chromatography (TLC) using silica gel plates (5735 silica gel 60F$_{254}$; 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 [^{14}C]-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 100ºC 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 CDCl$_3$ on a Bruker DRX500 operating at 500.13 MHz for ^{1}H-NMR and 125.77 MHz for ^{13}C-NMR.

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21

22

23

1   **FIGURE LEGENDS**

2   **Figure 1 Mycolic acid reductases in mycobacteria**

3   (A)   Schematic representation of the post-FAS-II steps in mycobacterial mycolic acid

4   biosynthesis. AccD4 and AccD5 are acyl-CoA carboxylases while FadD32 is an acyl-AMP

5   ligase. It is yet unclear whether the reduction of the β-oxo group occurs while the mycolic acid

6   precursor in still attached to Pks13 or after release from Pks13 by a thioesterase. (B) Alignment

7   of amino acid sequences of Rv2509 and MSMEG4722 with 1cyd (PDB file for the structure of

8   *Mus musculus* carbonyl reductase with NADP and 2-propanol). α-helices and β-sheets are

9   indicated above the residues as coils and arrows respectively.   Residues essential for

10   NAD/NADP binding are indicated by triangles while the active site residue is indicated with a

11   star.

12

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

14   (A)   Map of the *MSMEG4722* region in the parental *M. smegmatis* strain mc^{2}155 and its

15   corresponding region in the Δ*MSMEG4722* mutant.   *res*, γδ resolvase site; *hyg*, hygromycin

16   resistance gene from *Streptomyces hygroscopicus*; *sacB*, sucrose counterselectable gene from

17   *Bacillus subtilis*.   Digoxigenin-labelled probes were derived from ~1kb upstream and

18   downstream flanking sequences that were used to construct the knockout plasmid, and are

19   indicated by thick lines with square ends. *Cla*I digested bands expected in a Southern blot are

20   indicated in roman numerals with sizes in brackets. The inset shows the Southern blot of *Cla*I

21   digested genomic DNA from the two strains with expected bands indicated by arrows.   (B)

1 Colonies of wild type (mc^{2}155), mutant ($\Delta MSMEG4722$) and complemented ($\Delta MSMEG4722$-C)

2 strains on TSB-agar (-) or TSB-agar + 0.05% Tween-80 (+). Colony growth shown in upper row

3 was obtained by inoculating 5μL of a broth culture on the agar plate while lower row shows

4 pictures of a single isolated colony of each strain. Scale bar =1mm. (C) Growth curve of wild

5 type (mc^{2}155), mutant ($\Delta MSMEG4722$) and complemented ($\Delta MSMEG4722$-C) strains in TSB.

6

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

8 (A) TLC analysis of MAMEs extracted from mc^{2}155, $\Delta MSMEG4722$, $\Delta MSMEG4722$-C and

9 $\Delta MSMEG4722$-CRv strains.   The rapidly migrating species observed in $\Delta MSMEG4722$ are

10 indicated by a question mark. FAMEs; fatty acid methyl esters. (B) 2D Ag$^+$-TLC of MAMEs

11 extracted from mc^{2}155, $\Delta MSMEG4722$, $\Delta MSMEG4722$-C. UFAMEs, unsaturated fatty acid

12 methyl esters. (C) TLC analysis of MAMEs extracted from cells pre-treated with NaBH$_4$.

13

14 **Figure 4   2D-TLC analysis of apolar lipids extracted from mc^{2}155, $\Delta MSMEG4722$ and**

15 **$\Delta MSMEG4722$-C strains**
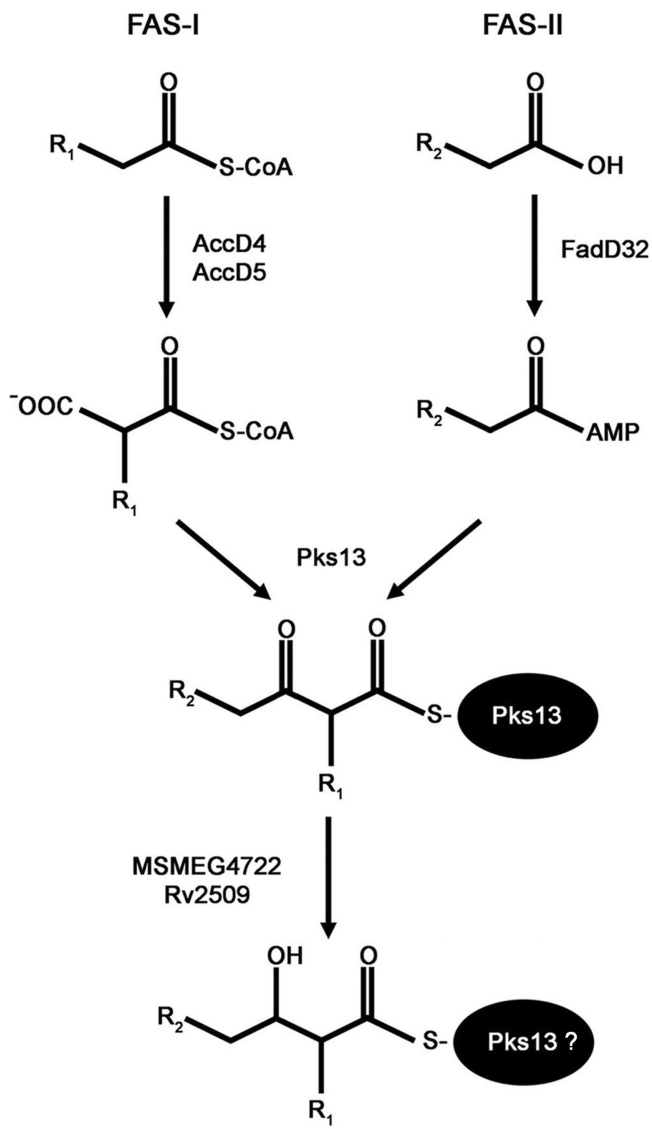
16 Panel (A), direction 1, chloroform:methanol:water, 100:14:0.8 (v/v); direction 2,

17 chloroform:acetone:methanol:water, 50:60:2.5:3 (v/v).   Panel (B), direction 1, petroleum

18 ether:acetone, 98:2 (v/v, thrice); direction 2, toluene:acetone, 98:2 (v/v). Panel (C), direction 1,

19 chloroform:methanol, 96:4 (v/v); direction 2, toluene:acetone, 80:20 (v/v). TMM, trehalose

20 monomycolate; TDM, trehalose dimycolate; GMM, glucose monomycolate; FA, fatty acids;

21 MA, mycolic acids. Lipid-Y is indicated by an arrow and question mark in Panel (B).

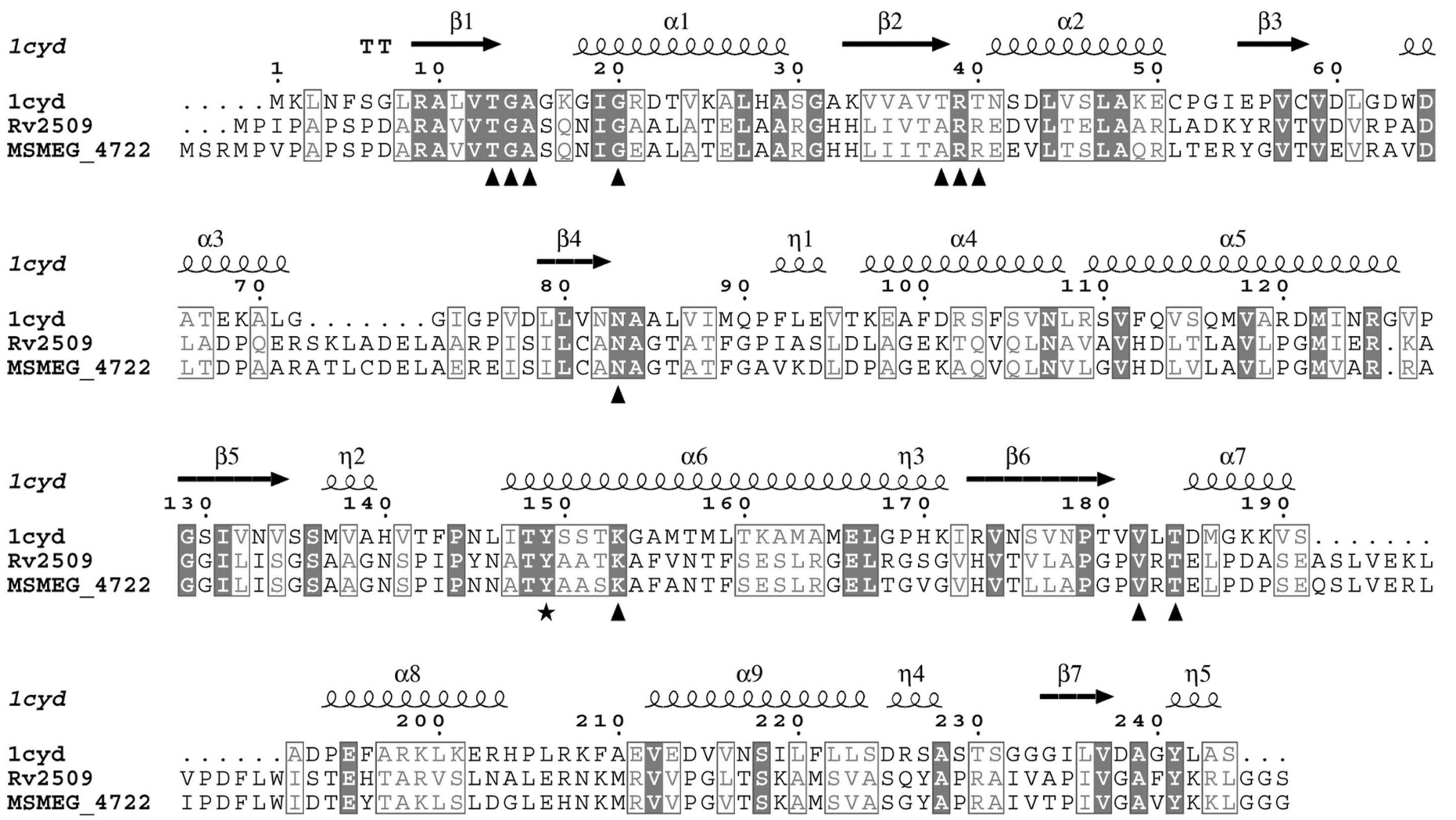1   **Figure 5 Structural analysis of Lipid-Y**

2   (A) MALDI-TOF/MS (B) ^{1}H-NMR and (C) ^{13}C-NMR of purified Lipid-Y. Characteristic shifts

3   are labelled as numbers and the structures they represent are indicated in (D).
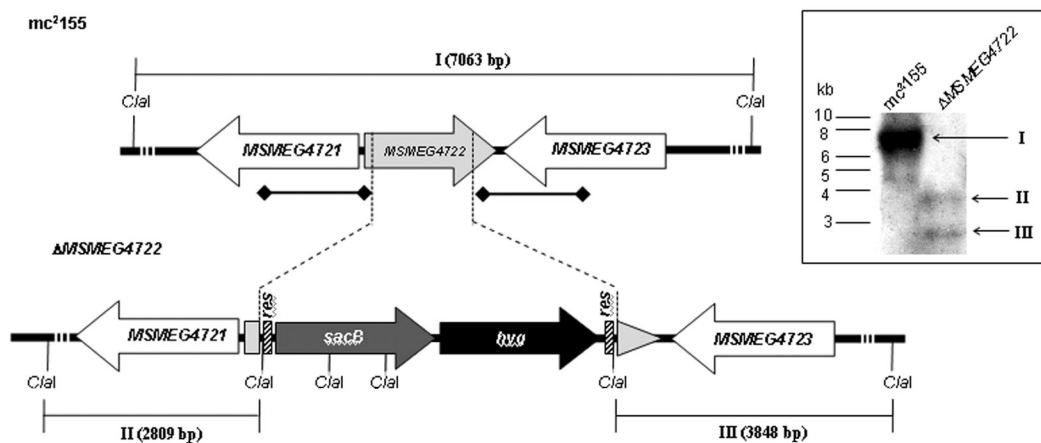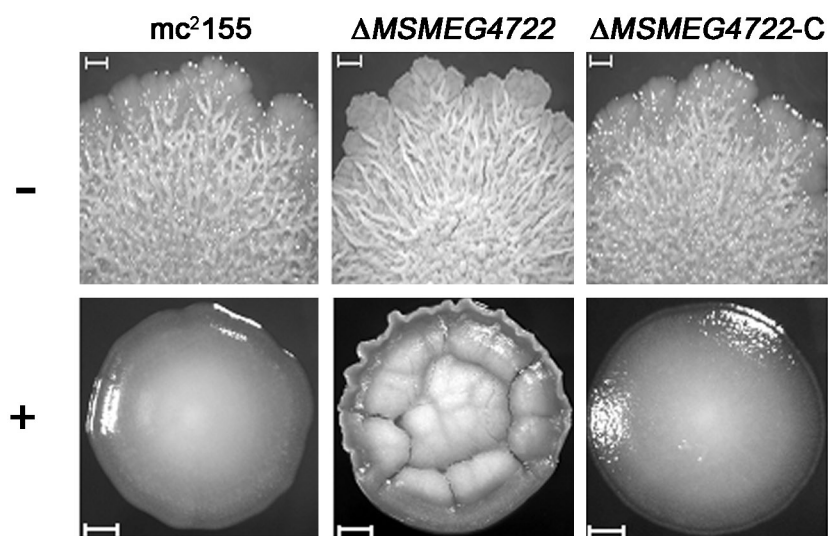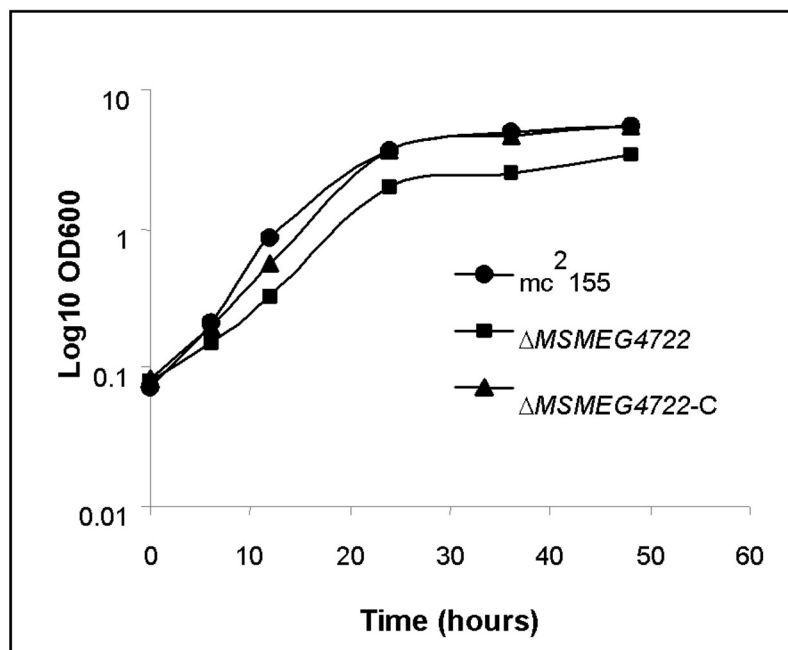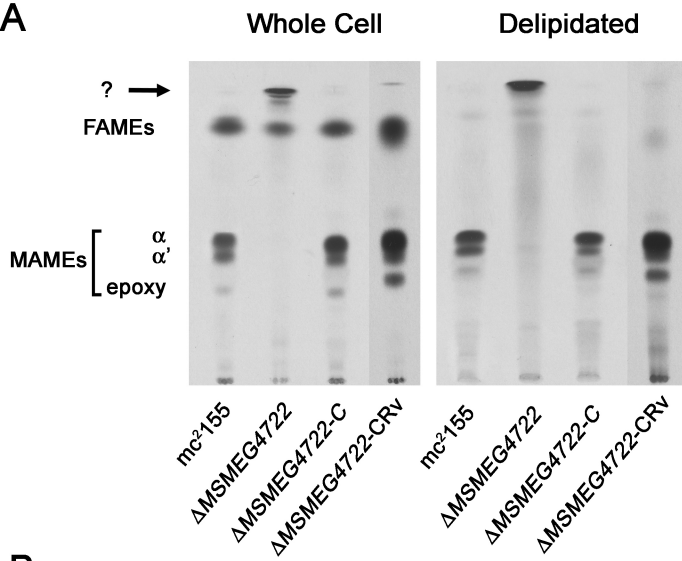
4

A

FAS-I

R_1–C(=O)–S-CoA

↓ AccD4 / AccD5

^-OOC–C(=O)–S-CoA (R_1)

FAS-II

R_2–C(=O)–OH

↓ FadD32

R_2–C(=O)–AMP

↓ Pks13

R_2–...–C(=O)–S– Pks13 (R_1)

↓ MSMEG4722 / Rv2509

OH / R_2–...–C(=O)–S– Pks13 ? (R_1)

B

```
1cyd              TT    β1              α1              β2           α2            β3
                        1      10        20        30        40        50        60
1cyd        .....MKLNFSGLRALVTGAGKGIGRDTVKALHASGAKVVAVTRTNSDLVSLAKECPGIEPVCVDLGDWD
Rv2509      ...MPIPAPSPDARAVVTGASQNIGAALATELAARGHHLIVTARREDVLTELAARLADKYRVTVDVRPAD
MSMEG_4722  MSRMPVPAPSPDARAVVTGASQNIGEALATELAARGHHLIITARREEVLTSLAQRLTERYGVTVEVRAVD
                     ▲▲▲    ▲                        ▲▲▲

1cyd            α3          β4        η1          α4              α5
                70        80        90       100       110       120
1cyd        ATEKALG.......GIGPVDLLVNNAALVIMQPFLEVTKEAFDRSFSVNLRSVFQVSQMVARDMINRGVP
Rv2509      LADPQERSKLADELAARPISILCANAGTATFGPIASLDLAGEKTQVQLNAVAVHDLTLAVLPGMIER.KA
MSMEG_4722  LTDPAARATLCDELAEREISILCANAGTATFGAVKDLDPAGEKAQVQLNVLGVHDLVLAVLPGMVAR.RA
                          ▲

1cyd              β5       η2          α6             η3      β6          α7
                130       140       150       160       170       180       190
1cyd        GSIVNVSSMVAHVTFPNLITYSSTKGAMTMLTKAMAMELGPHKIRVNSVNPTVVLTDMGKKVS.......
Rv2509      GGILISGSAAGNSPIPYNATYAATKAFVNTFSESLRGELRGSGVHVTVLAPGPVRTELPDASEASLVEKL
MSMEG_4722  GGILISGSAAGNSPIPNNATYAASKAFANTFSESLRGELTGVGVHVTLLAPGPVRTELPDPSEQSLVERL
                              ★    ▲                                ▲▲

1cyd                  α8                α9           η4       β7      η5
                    200       210       220       230       240
1cyd        ......ADPEFARKLKERHPLRKFAEVEDVVNSILFLLSDRSASTSGGGILVDAGYLAS...
Rv2509      VPDFLWISTEHTARVSLNALERNKMRVVPGLTSKAMSVASQYAPRAIVAPIVGAFYKRLGGS
MSMEG_4722  IPDFLWIDTEYTAKLSLDGLEHNKMRVVPGVTSKAMSVASGYAPRAIVTPIVGAVYKKLGGG
```

A

mc²155

I (7063 bp)

ClaI ... ClaI

MSMEG4721   MSMEG4722   MSMEG4723

ΔMSMEG4722

MSMEG4721 res sacB hyg res MSMEG4723

ClaI   ClaI   ClaI   ClaI   ClaI   ClaI

II (2809 bp)   III (3848 bp)

kb
10
8 I
6
5
4 II
3 III

mc²155   ΔMSMEG4722

B

mc²155   ΔMSMEG4722   ΔMSMEG4722-C

−

+

C

Log10 OD600 vs Time (hours)

- ● mc²155
- ■ ΔMSMEG4722
- ▲ ΔMSMEG4722-C

A

TMM
GMM
TDM

mc²155

ΔMSMEG4722

ΔMSMEG4722-C

B

mc²155

ΔMSMEG4722   ←?

ΔMSMEG4722-C

C

MA

mc²155

ΔMSMEG4722

ΔMSMEG4722-C
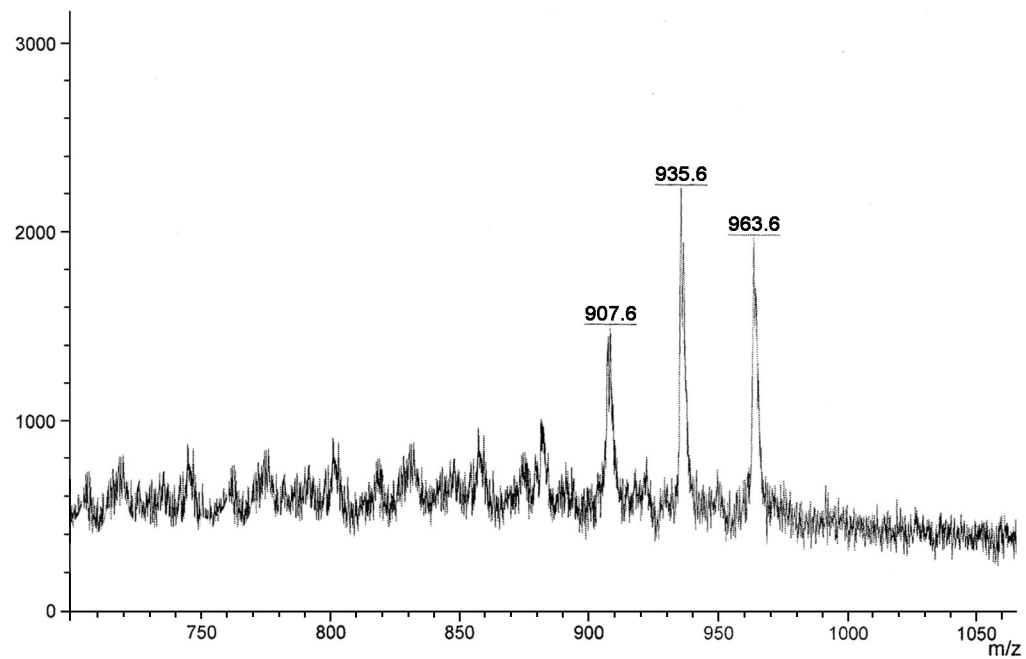
Table 1   Plasmids, bacterial strains, and phages used in this study

	Description	Reference/source
Plasmids		
pMV261	*E. coli-Mycobacterium* shuttle plasmid vector with *hsp60* promoter and Kan^{R}cassette (*aph*)	(Stover *et al.*, 1991)
pMV261-*MSMEG4722*	*MSMEG4722* cloned in pMV261	This work
pMV261-*Rv2509*	*Rv2509* cloned in pMV261	This work
p0004s	Vector for cloning allelic-exchange substrates to be used for specialized transduction; contains λ phage *cos* site and Hyg^R cassette (*hyg*)	Gift from T. Hsu and W.R.Jacobs Jr., Albert Einstein College of Medicine, New York
pΔ*MSMEG4722*	Derivative of p0004s designed for allelic exchange of *M. smegmatis MSMEG4722*	This work
Bacterial strains		
mc^{2}155	Electroporation-proficient *ept* mutant of *M. smegmatis* strain mc^{2}6	(Snapper *et al.*, 1990)
Δ*MSMEG4722*	Deletion mutant of mc^{2}155 in which *MSMEG4722* is replaced by *hyg*	This work
Δ*MSMEG4722*-C	Complemented strain of Δ*MSMEG4722* containing pMV261-*MSMEG4722*	This work
Δ*MSMEG4722*-CRv	Complemented strain of Δ*MSMEG4722* containing pMV261-*Rv2509*	This work
Phages		
phAE159	Conditionally replicating shuttle phasmid derived from the lytic mycobacteriophage TM4	(Bardarov *et al.*, 2002)
phΔ*MSMEG4722*	Derivative of phAE159 obtained by cloning pΔ*MSMEG4722* into its unique *Pac*I site	This work

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.

			Total Carbon number of mycolic acid								
Untreated			62	64	74	75	76	77	78	79	80
	WT	α'	952	980							
		α			1118	1132	1146	**1160**	1174	**1188**	
		epoxy				1148	1162	**1176**	1190	**1204**	1218
NaBH₄ treated											
	WT	α'	952	980							
		α			1118	1132	1146	**1160**	1174	**1188**	
		epoxy					1162	**1176**	1190	1204	
		hydroxyl						1178		1206	1220
	ΔMSMEG4722	α'	952	980							
		α			1118		1146	**1160**		**1188**	
		α-epi						**1160**		**1188**	
		epoxy						**1176**	1190		
		hydroxyl						1177			
	ΔMSMEG4722-C	α'	952	980							
		α			1118	1132	1146	**1160**	1174	**1188**	
		epoxy					1162	**1176**	1190	1204	
		hydroxyl						1178		1206	1220