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Role of the Mce1 transporter in the lipid homeostasis of *Mycobacterium tuberculosis*

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Abstract

Tuberculosis is one of the leading causes of mortality throughout the world. *Mycobacterium tuberculosis*, the causative agent of human tuberculosis, has developed several strategies involving proteins and other compounds known collectively as virulence factors to subvert human host defences and invade the human host. The Mce proteins are among these virulence-related proteins and are encoded by the *mce1, mce2, mce3* and *mce4* operons in the genome of *M. tuberculosis*. It has been proposed that these operons encode ABC-like lipid transporters; however, the nature of their substrates has only been revealed in the case of the Mce4 proteins. Here we found that the knockout of the *mce1* operon alters the lipid profile of *M. tuberculosis* H37Rv and the uptake of palmitic acid. Thin layer chromatography and liquid chromatography-mass spectrometry analysis showed that the *mce1* mutant accumulates more mycolic acids than the wild type and complemented strains. Interestingly, this accumulation of mycolic acid is exacerbated when bacteria are cultured in the presence of palmitic acid or arachidonic acid. These results suggest that the *mce1* operon may serve as a mycolic acid re-importer.

Keywords

mce operon; *Mycobacterium tuberculosis*; lipids; mycolic acid

Introduction

Tuberculosis is still one of the leading causes of mortality throughout the world. The HIV/AIDS pandemic, the deterioration of public health systems in developing countries and the emergence of multidrug-resistant forms of tuberculosis have further contributed to this situation. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, achieves

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infection by strategies involving the uptake and replication of the bacterium in host macrophages and the weakening or modulation of the host immune response [1]. The genome of *M. tuberculosis* contains four *mce* operons (*mce1–4*) with a similar arrangement within the operon [2]. These operons each comprise eight genes whose organization follows a similar pattern: two *yrbE* genes (A and B) are followed by six *mce* genes (A, B, C, D, E and F). The proteins encoded by these operons are homologous to ATP-binding cassette transporters (ABC-transporters) and present the typical gene arrangement of importers in which YrbEs are homologous to permeases and have a region within the penultimate cytoplasmic loop that may serve as the site of interaction to ATPases, whereas Mces are homologous to substrate-binding proteins (SBP) [3]. Several groups, including ours, have reported the relevance of Mce proteins during the replication of *M. tuberculosis* in mice [4–6]. Additionally, it has been suggested that MceA plays a role in the entry of the pathogen into non-phagocytic cells [7].

The completion of the genome sequences of *Mycobacterium avium* and *Mycobacterium smegmatis* indicates that these opportunistic species possess orthologs of the *mce* genes. Moreover, *mce* operons with an identical structure have been identified in all *Mycobacterium* species examined and in five other species of the genus Actinomycetales [3]. On the basis of conserved domains, previous studies have suggested that the *mce* and *yrbE* genes encode components of transport systems [3, 8] involved in the translocation of lipids. In line with this, it has been demonstrated that the *mce4* operon encodes a cholesterol import system that enables *M. tuberculosis* to derive both carbon and energy from this host lipid [9]. Interestingly, Kendall et al. [10] have shown that *mce4* is regulated by KstR, a TetR-type regulator, along with other genes involved in fatty acid metabolism. In addition, we have found that *mce3R*, a regulator of the *mce3* operon, regulates the expression of proteins involved in lipid metabolism [11]. Evidence to date thus point to the *mce* operons encoding transporters of lipids or related compounds.

The fact that multiple copies of these potential lipid transporters are present in mycobacterial genomes is consistent with the finding that pathogenic mycobacteria switch from carbohydrates to lipids as their main carbon and energy source inside cells. This interchange between different sources most likely requires the activity of multiple lipid or fatty acid import systems. This is also supported by the observation that the expression of *mce3* is down-regulated *in vitro* [12] and that *mce1* is up-regulated inside host cells [13].

A *M. tuberculosis* mutant deficient in the expression of one of the *mce1* genes displayed intracellular growth defects, indicating that the Mce1 transporter is required for optimal replication inside host cells The intracellular requirement of the Mce1 proteins is in agreement with the fact that it is the only Mce operon conserved in the obligate pathogen *Mycobacterium leprae*, in spite of its genome reduction. *mce1* is also the most conserved of all *mce* operons across the *Mycobacterium* genus with orthologs in both slow and fast-growing species including *Mycobacterium smegmatis*. The role of *mce1* in *M. tuberculosis* virulence is, however, still controversial. Surprisingly, a *M. tuberculosis mce1* mutant was found to be hypervirulent when administered intravenously or intraperitoneally, whereas it displayed an attenuated phenotype when administered intratracheally [5, 6]. Therefore, despite recent advances in deciphering the functions of Mce proteins as a whole, the precise substrate transported by the Mce1 proteins and the contribution of this transporter to intracellular growth are still poorly understood. Gaining insight into the function of this transporter would be key to the understanding of its contribution to the adaptation of *M. tuberculosis* to the host environment. In this work, we investigated the role of the Mce1 proteins in the import of fatty acids, particularly palmitic acid, and the effect of a deficiency in these proteins on the lipid composition of *M. tuberculosis*.

Material and Methods

Bacterial strains, media and growth conditions

All cloning steps were performed in *Escherichia coli* DH5α grown either in Luria-Bertani (LB) broth or on LB agar at 37°C.

M. tuberculosis and *Mycobacterium smegmatis* mc^{2}155 were grown in Middlebrook medium supplemented with albumin 0.5%, dextrose 0.4%, glycerol 0.5% (7H9-ADG) or Middlebrook 7H11 supplemented with albumin, dextrose and glycerol (7H10-ADG). For growth on glycerol, Sauton's minimal medium (0.5 g/liter $K_2HPO_4$0.5 g/liter $MgSO_4$. $7H_2O$, 2 g/liter citric acid, 4 g/liter L-asparagine, 0.05 g/liter ferric ammonium citrate, pH 6.9) supplemented with glycerol 0.5% was used. For growth on fatty acids as carbon source, modified Sauton's medium with low asparagine (0.4 g/liter) was supplemented with 0.5% fatty acid free albumin (Sigma-Aldrich Co.) and 30 µM palmitic acid or 50 µM arachidonic acid. When required, 50 µg/ml hygromycin, 20 µg/ml kanamycin, Tween 80 0.05% or 0.025% (vol/vol) tyloxapol were added to the medium.

Mycobacterium electrocompetent cells were prepared as described by Wards and Collins [14].

Complementation of the *mce1* mutant

The *M. tuberculosis* Δmce1 mutant was constructed by insertion of a hygromycin cassette within the *yrbE1B* gene [5]. The mutation causes a polar effect abolishing the transcription downstream genes. Specifically, RT-qPCR analyses showed that the mutation reduces 52 folds ($p<0.05$) the transcription of *mce1B*, while the transcription of the gene *masA*, which is located downstream of *mce1F*, was equivalent in the mutant and in the wild type strain (fold change 1.2, $p<0.05$). Complementation of the Δmce1 mutant was carried out with the entire *mce1* operon encompassing *yrbE1A-mce1F*. The entire *mce1* operon was amplified from *M. tuberculosis* H37Rv genomic DNA as two separate fragments. The *mce1* upstream fragment was amplified using the primers: upNcomce1C: 5′CCATGGTCTGCATCAGCTGGTTTCAC3′ and dwNcomce1C: 5′CCATGGTCGATCTCGAGCACTTTCCT3′. The primers carry an *Nco*I restriction site (underlined). The resulting 4657 bp-PCR product carries an internal *Hind*III site. The *mce1* downstream fragment was amplified using the following primers: upHindIIImce1C: 5′AAGCTTTACCAGTGTTCCCATGCTAT3′ and dwHindIIImce1C: 5′AAGCTTCCTTCACCTCCGTAGACGAC3′, both carrying a *Hind*III restriction site (underlined). The 4657 bp-upstream fragment was cloned into the *Nco*I site in the integrative pYUB178 vector, giving rise to the pYUB178::mce1up recombinant vector. The 5806 bp-downstream DNA fragment was then cloned into the internal *Hind*III site of pYUB178::mce1up, giving rise to pMtmce1, the vector carrying the entire *mce1* operon used in complementation studies. The complemented mutant strains were selected on kanamycin-containing medium. The presence of pMtmce1 in the complemented mutant strain was verified by PCR.

Fatty acid import assay

M. tuberculosis strains were grown in Middlebrook 7H9-ADGT at 37°C until exponential phase. Cells were washed and diluted to ~ OD_{600nm} of 0.6–0.8 in 7H9-ADGT or Sauton low asparagine medium supplemented with tyloxapol (minimal medium). Heat-killed cells were used as a negative control in order to estimate the amount of radioactivity unspecifically adsorbed to the cells. Lipid uptake experiments were performed as described by Pandey and Sassetti [9] with 0.02 µCi/ml [^{14}C(U)]-palmitic acid. The bacteria were incubated at 37°C and 1-ml culture samples were taken at different time points. Cells were collected on a 0.4-

µm Millipore filter and radioactivity counted using a 1450 Microbeta TriLux Liquid Scintillation and Luminescence Counter (Perkin Elmer).

Lipid analysis

Total lipids from bacterial cells and culture filtrates were extracted following procedures described earlier [16] and analyzed by TLC on silica gel $60F_{254}$ loading the same lipid quantities per lane (300 µgr). TLCs were developed using chloroform:methanol:water (90:10:1) or (60:30:6); η-hexane:ethyl acetate (95:5 v/v) (thrice) and revealed by spraying with a $CuSO_4$-phosphoric acid solution and heating.

Fatty acid and mycolic acid methyl esters (FAMES and MAMEs, respectively) were derived from culture filtrate and extractable lipids and from cells as described by Stadthagen *et al.* 2005. FAMEs and MAMEs were developed three times into *n*-hexane:ethyl acetate (95:5 v/v).

Liquid chromatography-mass spectrometry (LC-MS) analysis

Total lipids from *M. tuberculosis* H37Rv and the Δmce1 mutant grown on minimal Sauton medium supplemented with palmitic acid as the carbon source were analysed by LC/MS as described by Sartain et al. [17]. A high resolution Agilent 6220 TOF mass spectrometer interfaced to a LC was used. Data files were analyzed with Agilent's Mass hunter workstation software (Version B.02.00, build 2.0.197.0) to identify compounds using 'molecular feature extractor'. The Agilent mass profiler program was used to compare lipids and mycolic acids present in the sample. Most compounds were identified using the lipid database developed by [17]. Compounds of interest were semi-quantified by comparing their relative abundance in the samples.

qRT-PCR

qRT-PCR reactions were performed as described by Santangelo et al [11] and Blanco et al [18]. DNA-free RNA (1µg) was extracted from mid-exponential cultures grown in 7H9-ADCT. The primers used are listed in the Supplementary data 1. Relative quantification was performed by using *sigA* as a reference and subsequent analysis for statistical significance was as described by Blanco et al [18].

Protein expression and immunoblot analysis

The *yrbE1B*, *mce1A*, *mce1B*, *mce1C* and *mce1E* genes from the *mce1* operon were amplified individually from *M. tuberculosis* H37Rv genomic DNA using the sets of primers listed in Supplementary data 1. The PCR fragments were then cloned into the expression vector pML2031 (kindly provided by Dr. M. Niederweis). pML2031 allows for the nitrile-inducible expression of genes and production of C-terminal-fused hemagglutinin (HA)-proteins. Recombinant vectors were electroporated into *M. smegmatis* mc^2155. Strains were grown on 50 ml of Hyg-containing LB until exponential phase, and the expression was then induced by adding 500 µM isovalernitrile over night. Cells were collected, disrupted by using a bead beater and centrifuged at 6,000g for 10 min. The supernatants were centrifuged at 23,000g for 30 min. The pellets enriched in cell wall proteins were resuspended in 50 µl of saline phosphate buffer (PBS) and supernatants were centrifuged at 100,000g for 2hs30 min. The pellets enriched in cell membrane proteins were resuspended in 50 µl of PBS. The supernatants containing cytoplasmic proteins were precipitated with 10% trichloroacetic acid, washed twice with cold acetone and resuspended in 50 µl of PBS. The protein extract were resolved in a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes (Hybond ECL 0.45µm GE Healthcare). The localization of the YrbE1 and Mce1 proteins was analysed by Western blot using a monoclonal anti-HA (Sigma-Aldrich, H9658) primary antibody diluted 1:5,000; and an anti-IgG mouse peroxidase-tagged (Sigma-Aldrich, A9044) secondary antibody diluted 1:10,000. Immune complexes were visualized using a BCIP/NTP solution (Promega).

Results

Mutating the *mce1* operon affects the uptake of palmitic acid by *M. tuberculosis*

To investigate the involvement of Mce1 proteins in the transport of fatty acids, we comparatively analysed the incorporation of radiolabelled palmitic acid by the wild type, mutant and complemented mutant strains. Figure 1A shows that the uptake of palmitic acid was less pronounced in the mutant strain than in the wild type and complemented mutant strains with significant differences at 2 and 4 hs ($p<0.05$). Importantly, *M. tuberculosis mce3* mutant showed equivalent incorporation of palmitic acid to that of the wild type strain, indicating that the observed deficiencies in the uptake of palmitic acid is specific to the *mce1* mutation (Supplementary data 2).

In order to test if the Mce1 transporter is essential for the uptake of palmitic acid, we evaluated the growth of a *M. tuberculosis* Δmce1 mutant in the presence of this fatty acid as the unique carbon source. The Δ*mce1* mutant showed no significant alterations of growth under this condition (Figure 1B). This result may indicate either that the palmitic acid uptake rate in the mutant is sufficient to sustain normal growth or that other mechanisms are triggered for the uptake of this fatty acid. A repeat palmitic acid uptake experiment in a medium containing nanomolar concentrations of radiolabeled palmitic acid as the sole carbon source indicated a significantly less pronounced uptake of palmitic acid in the mutant strain compared to the wild type and complemented mutant strains at early time points, then, levels reached a plateau for all strains (Figure 1C), likely, because all radiolabeled palmitic acid was incorporated by cells after 60 min. As expected, *M. tuberculosis* incorporates more radiolabeled palmitic acid in this limited carbon source condition than in a carbon rich condition (Figure 1C).

The lipid profile of the *mce1* mutant is altered in the presence of palmitic acid as a carbon source

We next investigated the impact of the deletion of the *mce1* operon on the lipid composition of *M. tuberculosis*. When palmitic acid was used as the carbon source, the mutant strain consistently accumulated a putative lipid compounds that were restored to wild type levels in the complemented mutant strain (see LI in Figure 2). These phenotypes were reproducible throughout five independent experiments. Similarly, when bacteria were grown in glucose +glycerol medium, the LI content in the *mce1* mutant showed an increase comparing to the wild type strain (Supplementary data 3A). However, quantification of LI band intensities indicates that in the fatty acid supplemented medium the differences in LI content between the *mce1* mutant and the wild type strain were higher than in rich medium: The average percentages for the LI band intensity in the mutant strain relative to the wild type strain were: 2.85 ± 1.5 in palmitic acid medium and 1.19 ± 0.2 in glucose+glycerol medium.

Preparation and TLC analysis of fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) from the cellular extractable lipids pointed to a increase in the mycolic acid content of the Δ*mce1* mutant compared to the wild type and complemented mutant strains (Figure 3C and Supplementary data 3B). This observation was confirmed by LC-MS analysis of the cellular extractable lipids from the wild type and mutant strains (Figure 3A). Mass spectra clearly showed an increase in the peaks (~ 5 folds) eluting between 22 and 24 min matching with free alpha, methoxy and ketomycolates in the Δ*mce1* mutant (Figure 3B).

It has been reported previously that the growth of a mutant in *fadD5* (a member of the *mce1* operon) of *M. tuberculosis* is slightly affected when bacteria are cultured in mycolic acid as sole carbon source, suggesting that mycolic acids may serve as substrates for Mce1 [19]. We thus analysed by TLC the mycolic acid content of the culture filtrates from the WT, mutant and complemented mutant strains. TLC analysis of the MAMEs prepared from culture filtrate lipids indicated that this content was increased in the *mce1* mutant compared to the wild type and complemented mutant strains (Figure 3D).

The lipid profile of the *mce1* mutant is altered in the presence of arachidonic acid as a carbon source

A search on the TBdatabase (http://www.tbdb.org) showed that the expression of some *mce1* genes is altered in the presence of either palmitic or arachidonic acids or both. For this reason, we also analyzed the lipid profile of the mutant, wild type and complemented strains in minimal medium supplemented with 50 μM arachidonic acid. After two months of culturing all *M. tuberculosis* strains reached an optical density (OD_{600nm}) lower than 0.2, indicating that *M. tuberculosis* grows poorly on arachidonic acid as carbon source (data not shown). The lipid profile of the mutant grown under this culture condition appeared similar to that of the bacteria grown in palmitic acid when resolved in the solvent system chloroform: methanol: water 90:10:1 (Figure 4A and B and 2A and B), showing accumulation of the LI compound in the cellular extractable and supernatant fractions. Remarkably, in the culture supernatant lipid fraction, the mycolic acid content was increased in the Δmce1 mutant as compared to the wild type and complemented strains (Figure 3F). However, in the cellular extractable lipid fraction this mycolic acid accumulation was not as obvious as in culture supernatant lipid fraction of the mutant strain (Figure 3E). Surprisingly, the complemented mutant strain showed a significant decrease in mycolic acid content compared to both the wild type and complemented mutant strains in arachidonic culture condition. Although the reason of this diminished content of mycolic acid in the complemented mutant strain is not clear, we speculate that in this strain, the negative regulation of *mce1* operon exerted by the Mce1 transcriptional repressor is unbalanced due to presence of an additional copy of *mce1* promoter/regulation region (one copy is in the wild type *locus* and other is in the integrative plasmid). As a consequence, more Mce1 proteins would be produced, resulting in less accumulation of mycolic acids.

In order to determine whether or not the LI compounds were free mycolic acids, cellular extractable lipids from bacteria grown in different carbon sources were solved in preparative TLCs and the materials migrating at LI position were purified from the silica and treated as they were mycolic acids. The lipid profile of all LI fractions showed a typical mycolic acid pattern (Figure 5).

Altogether these results indicate that the Mce1 proteins are important for the homeostasis of free mycolic acid of *M. tuberculosis*.

Most of the Mce1 proteins localize in the cell wall

Based on bioinformatics predictions, Casali et al. [3] proposed that the YrbE1AB and Mce1A-F proteins constitute the permeases and SBP of an ABC transporter, respectively, with the protein Mkl (Rv0655) being the ATPase providing the energy for metabolite translocation. In addition, Song et al. [20] proposed that the *mceA-F* genes of *M. tuberculosis* encode outer membrane proteins (OMPs) which form an outer membrane complex. Based on these predictions, it has been hypothesized that the Mce proteins localized to the surface and/or into the cell wall. However, until now, only Mce1A has been shown to localize at the surface of the bacterium [21]. In order to gain insight into the localization of the proteins encoded in *mce1* operon, we expressed the proteins YrbE1B, Mce1A, Mce1B, Mce1C and Mce1E of *M. tuberculosis* as HA-fusion under an inducible promoter in *M. smegmatis*. Previous attempts to express *mce1* genes under a constitutive promoter in *M. smegmatis* or *M. tuberculosis* were unsuccessful (data not shown). Western blot analysis using anti-HA antibodies detected recombinant YrbE1B-HA exclusively in membrane-enriched fraction. Mce1A-HA, Mce1B-HA and Mce1E-HA proteins were present in the cell wall-enriched fractions but less intense bands were also detected in the membrane-enriched fractions (Figure 6). Similarly, an analysis of cytoplasm, cell membrane and cell wall proteome profiles of *M. tuberculosis* have identified Mce1A in cell membrane fraction [22]. Although the whole Mce1C-HA protein (53.8 KDa) is predominant in the cell wall-enriched fraction, very intensive low molecular weight bands, likely corresponding to degraded forms of the proteins, were detected in cell membrane fraction (Supplementary data 4A). Therefore, from these results it is not possible to determine the distribution of Mce1C in the cell envelope compartments.

Taken together, these results are consistent with the role proposed for Mce as a transporter localized in the mycobacterial cell envelope.

Discussion

Here we found that the mutation of the *mce1* operon in *M. tuberculosis* produced an increase in the amount of mycolic acids, mainly in the culture supernatant lipid fraction, together with the accumulation of putative lipid. Remarkably, this alteration in the mutant lipid profile was only substantial when the carbon source in the culture medium was palmitic or arachidonic acids. In addition, the *mce1* mutation in *M. tuberculosis* significantly affected the uptake of palmitic acid. The mutant's phenotypes suggest two possible scenarios.

In one scenario, the Mce1 system directly participates in the transport of palmitic acid. This role is consistent with the reduced uptake of this specific fatty acid in the mutant strain.

In the second-scenario, Mce1 system is involved in the lipid remodelling that occurs when *Mycobacterium* is cultivated in the presence of different carbon sources. This Mce1-driven lipid remodelling is more intensive in limited carbon sources or in the presence of fatty acids, such as palmitic or arachidonic acids. This is consistent with the quantitative alterations in the lipid profile of the mutant strain in palmitic and arachidonic acids media. This last scenario is supported by the finding that mycobacteria and other microbes recycle cell wall components as a strategy to preserve scarce resources [23]. Importantly, during the preparation of this paper, Cantrell et al. reported that free mycolic acids accumulate in a *mce1* mutant strain of *M. tuberculosis* [24], sustaining the results of this study. However, opposite to the Cantrell′s observations we did not detect major alterations in the free mycolic acid accumulation among strains when bacteria were grown in glycerol as carbon source. Differences in the glycerol-containing culture media and methodologies used in both studies may explain these dissimilar results. Kalscheuer et al. have found that the LpqY-SugA-SugB-SugC ATP-binding cassette transporter is a recycling system mediating the retrograde transport of released trehalose in *M. tuberculosis* and *M. smegmatis* [25]. These authors proposed a model in which the trehalose moieties released from trehalose monomycolate by mycobacteria upon mycolic acid transfer to cell envelope acceptors are recycled by the cells to continue serving as a carrier of mycolic acids to the periplasm or to serve as a carbon source during growth in nutrient-limited microenvironments *in vivo*. In addition, Dunphy et al. have demonstrated that mycolic acid can be used by *M. tuberculosis* as a carbon source [19]. In connection with that, Dunphy et al. study showed that a *M. tuberculosis* mutant in the putative fatty acid binding site of *fadD5*, a gene co-transcribed with *mce1* genes, showed decreased growth in minimal medium containing mycolic acid, thus suggesting that FadD5 participates in mycolic acid degradation. Dunphy's study has also shown that a *M.*

tuberculosis mce1 operon mutant (which expresses intact FadD5) was able to grow normally in mycolic acid [19], suggesting that in this growth condition others transporters participate in mycolic acid recycling. In this regard, it is important to note that we mainly detected mycolic acid accumulation when the mutant was grown in palmitic or arachidonic acids, a condition different from that used by Dunphy et al. [19]. In this scenario, we hypothesize that, similarly to trehalose recycling, free mycolic acids and other cell wall lipid compounds are recycled to be used as substrates of lipid biosynthesis and/or as carbon sources in starvation conditions. This is in agreement with the concept that pathogenic mycobacteria switch their metabolism from carbohydrate to lipid pathways during their intracellular life.

In this study we found that the Mce proteins localize mainly in the cell wall, which is consistent with a role as substrate binding proteins of importer systems. We also demonstrated for the first time that the lack of Mce1 proteins affects the cell wall lipid structure, impairing the fatty acid uptake. These results together with the finding that mycolic acids accumulated extracellularly in the Δmce1 mutant suggest that Mce1 may serve as a mycolic acid re-importer in limited conditions of growth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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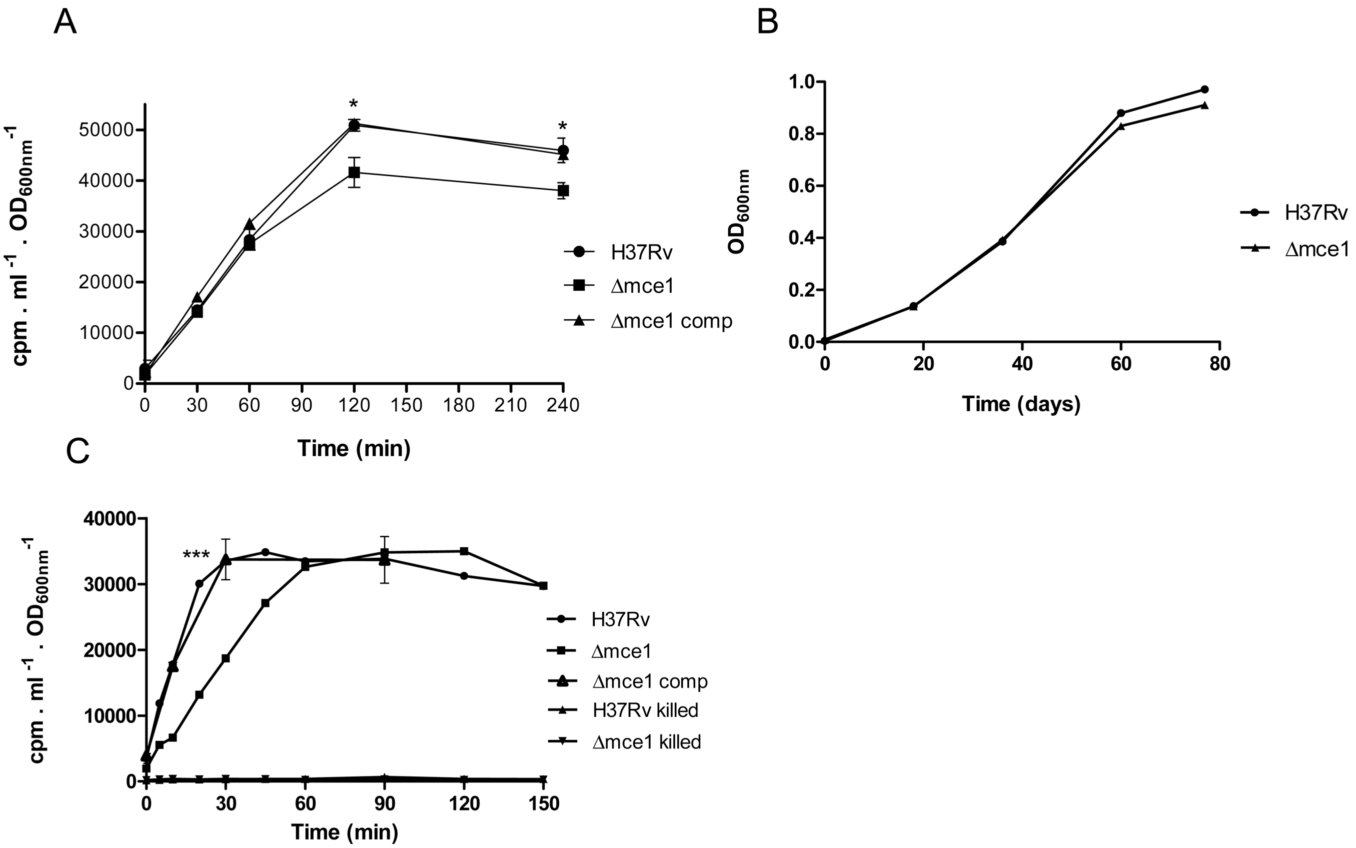


Figure 1. Palmitic acid uptake in *M. tuberculosis* strains

A) Palmitic acid uptake of *M. tuberculosis* H37Rv (H37Rv), the Δmce1 mutant (Δmce1) and the complemented mutant strain (Comp). The cells were grown in a rich medium (7H9-ADGT) until exponential growth phase and diluted at OD_{600nm} 0.6. The uptake was started by adding rich medium containing 0.02 μCi/ml [^{14}C]-palmitic acid. Aliquots of 1ml were taken at different time points and the amount of radioactivity associated to the cells was counted and expressed relative to the OD_{600nm} of the culture. B) Growth of *M. tuberculosis* H37Rv (H37Rv) and the Δmce1 mutant (Δmce1) in minimal medium with tyloxapol and supplemented palmitic acid as carbon source. C) Palmitic acid uptake of bacteria in minimal medium. The wild type (H37Rv, mutant (Δmce1) and complemented mutant (Comp) strains were grown in rich medium (7H9-ADGT) until exponential growth phase then were washed and suspended at OD_{600nm} 0.6 in low asparagine medium containing tyloxapol and 0.02 μCi/ml [14C]-palmitic acid. Aliquots were taken and counting. The cells exposed to 80°C for 1 hour (killed) were used as a measure of the background radioactivity bound to the cells. Statistically significant differences were observed between the wild type (H37Rv) and mutant (Δmce1) strains where (*) $p < 0.05$ and (***) $p < 0.001$. The graph 1A shows a representative experiment of two experiments performed in duplicate while the graph 1C shows a representative experiment of four experiments performed in duplicate.

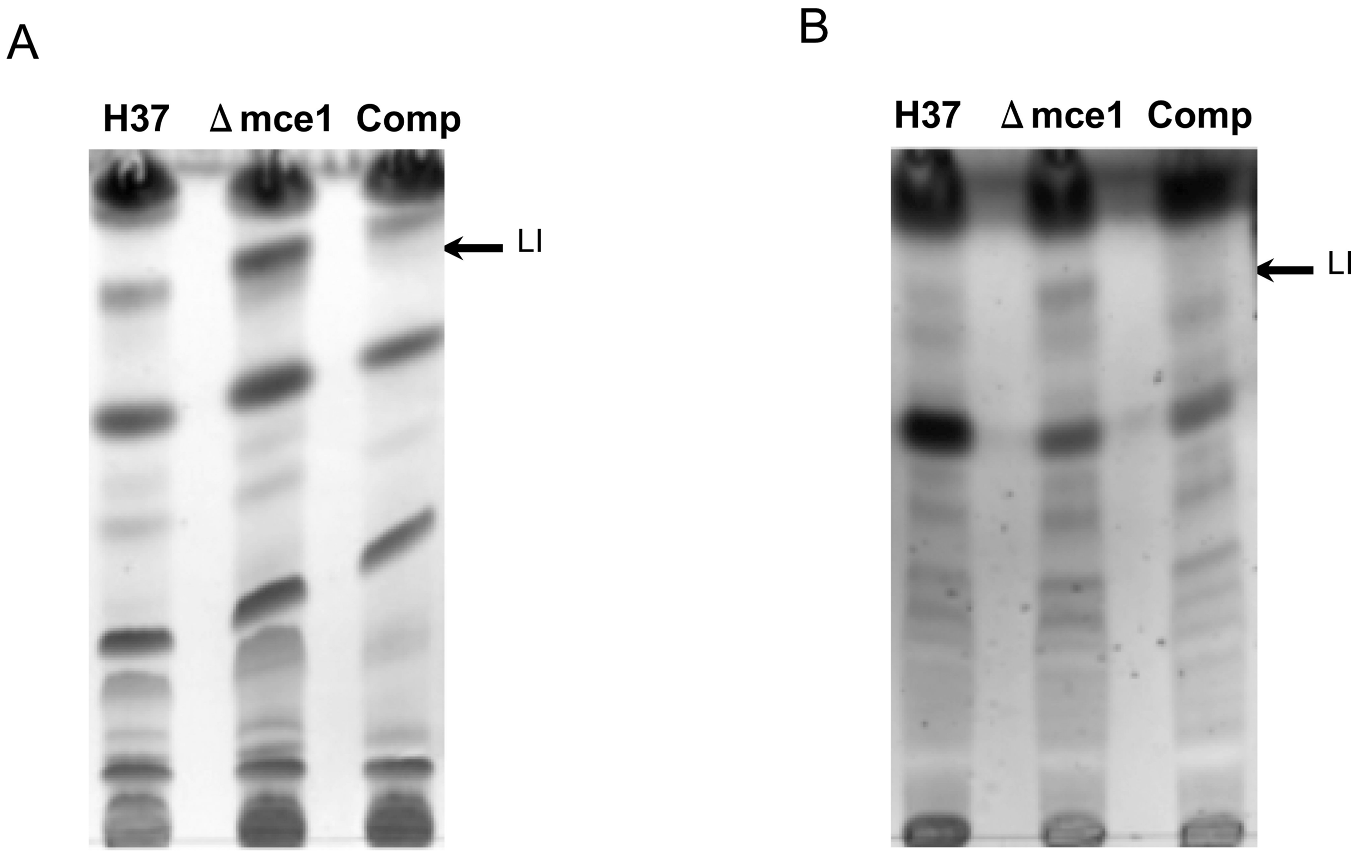
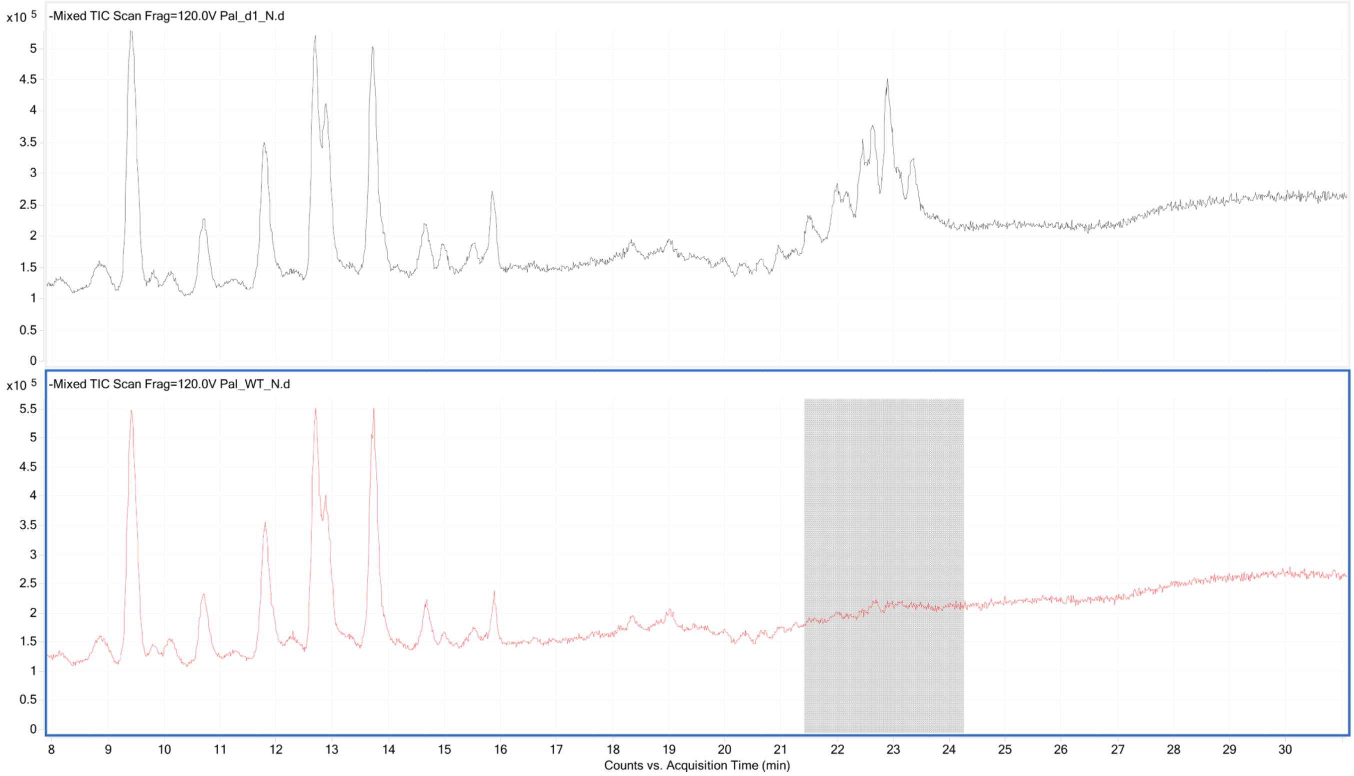


Figure 2. The lipid profile of the Δmce1 mutant is altered in response to palmitic acid as carbon source

Total lipids were extracted from *M. tuberculosis* H37Rv (H37), the Δmce1 mutant (Δmce1) and the complemented mutant strain (Comp) grown in minimal medium supplemented with palmitic acid. The lipids were developed by TLC in the solvent system chloroform:methanol:water (90:10:1) and revealed with CuSO4 and heating. The arrows indicate the LI increased lipids in the Δmce1 mutant. A) Cellular extractable lipids and B) Lipids from the culture supernatant. These lipid patterns were repeated in five independent experiments.

A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4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
x10 5
-Mixed TIC Scan Frag=120.0V Pal_WT_N.d
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
25
26
27
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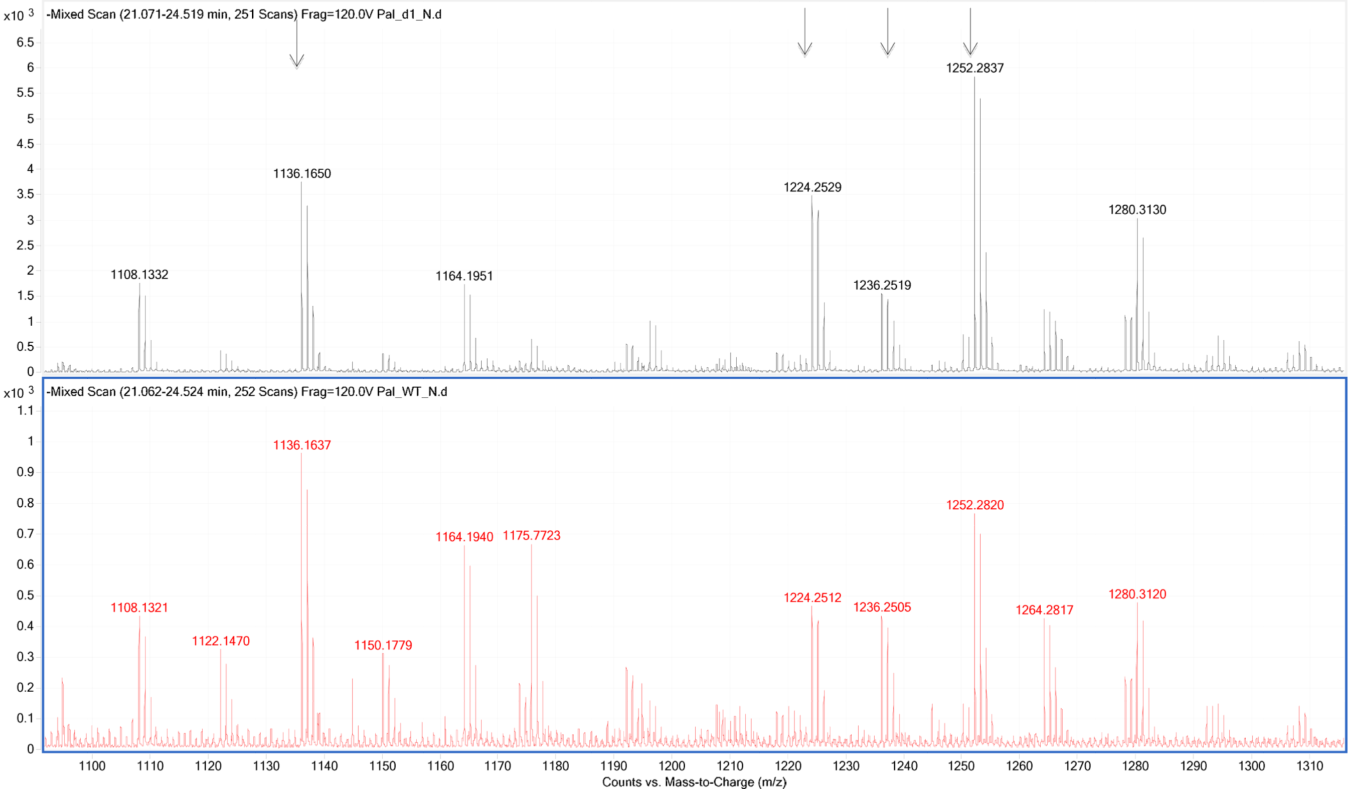
29
30
Counts vs. Acquisition Time (min)

B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6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1108.1332
1136.1650
1164.1951
1224.2529
1236.2519
1252.2837
1280.3130
x10 3
-Mixed Scan (21.062-24.524 min, 252 Scans) Frag=120.0V Pal_WT_N.d
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1108.1321
1122.1470
1136.1637
1150.1779
1164.1940
1175.7723
1224.2512
1236.2505
1252.2820
1264.2817
1280.3120
1100
1110
1120
1130
1140
1150
1160
1170
1180
1190
1200
1210
1220
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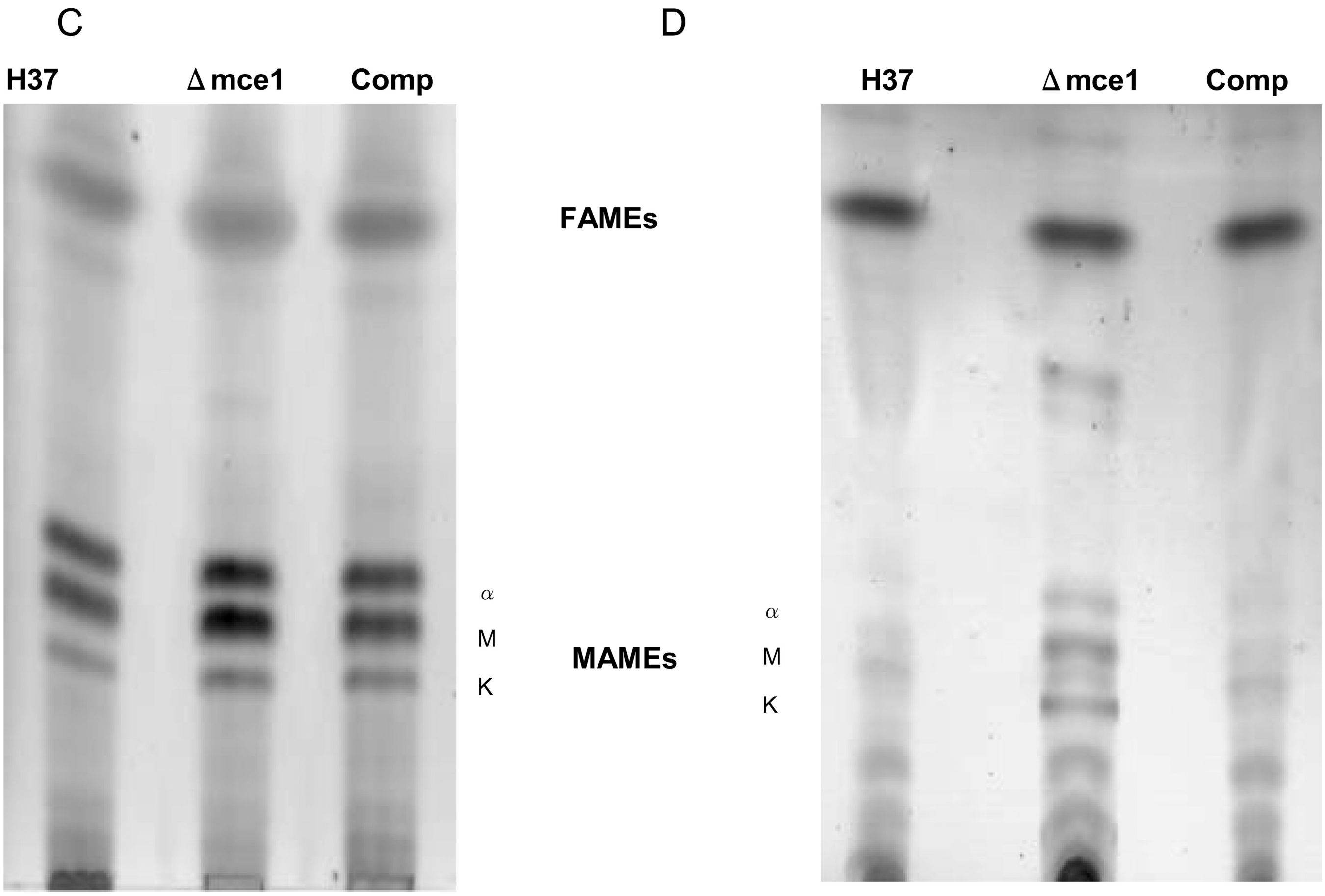
1240
1250
1260
1270
1280
1290
1300
1310
Counts vs. Mass-to-Charge (m/z)


C
H37
Δ mce1
Comp
FAMEs
α
M
K
MAMEs
D
H37
Δ mce1
Comp
α
M
K

NIH-PA Author Manuscript NIH-PA Author Manuscript NIH-PA Author Manuscript

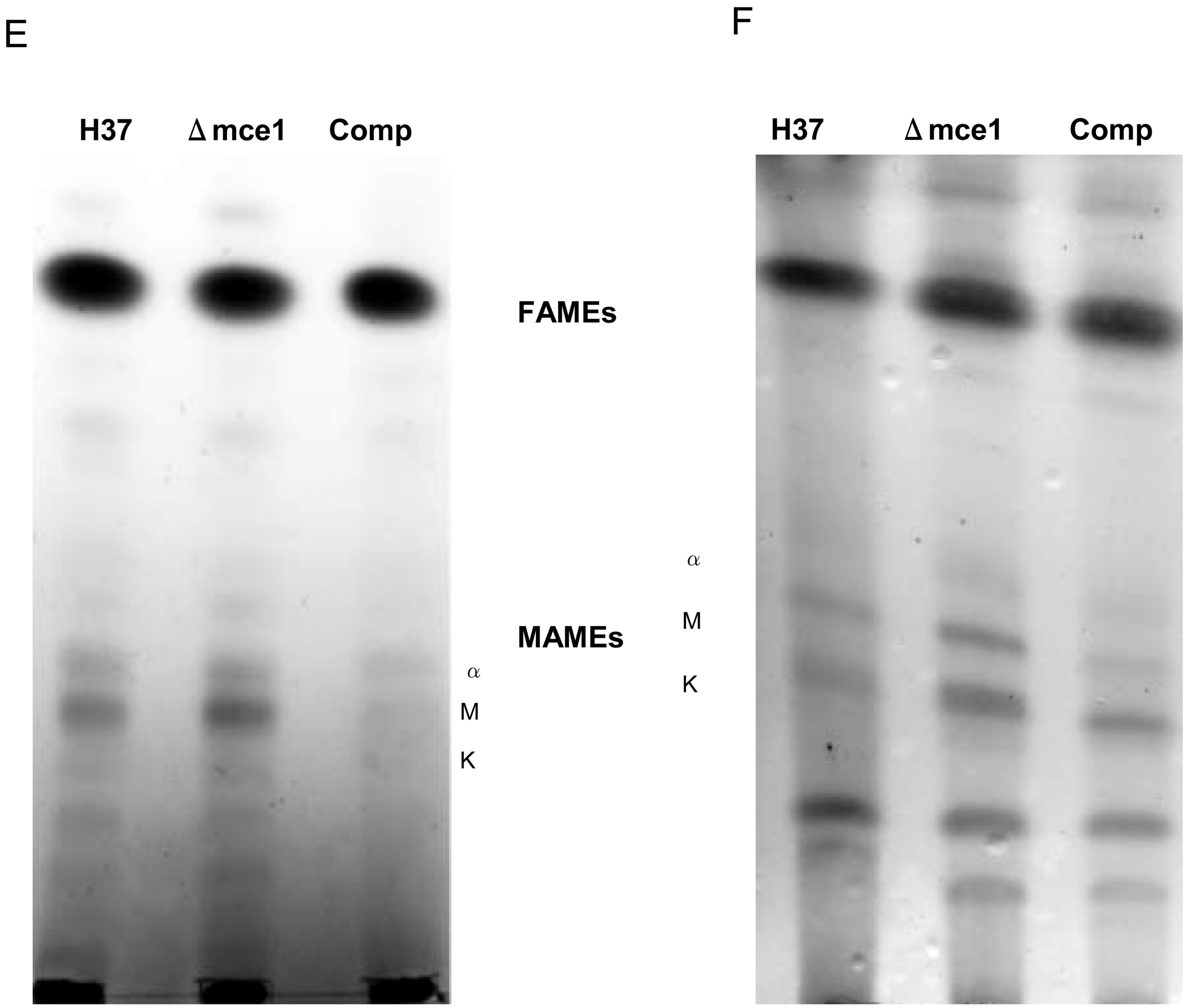


Figure 3. The mycolic acids are increased in the Δmce1 mutant

A) Chromatogram of liquid chromatography-mass spectrometry (LC-MS) of cellular lipids from *M. tuberculosis* strains showed an increased peaks in the Δmce1 mutant (top) in relation with the wild type H37Rv (bottom). Peaks between 9 and 16 minutes are cardiolipids. B) Mass spectra of grey area showed in A. The peaks indicated with arrows were identified by mass spectrometry as to free mycolic acids (not shown): 1136=C-78 alpha; 1224=C-83 methoxy 1236=C-84 keto 1252= C-84 methoxy. C, D, E, F) FAMEs and MAMEs prepared from cellular extractable (C, E) and lipids from supernatant (D, F) from *M. tuberculosis* H37Rv (H37), the Δmce1 mutant (Δmce1) and complemented mutant strain (Comp) cultured in minimal medium supplemented with palmitic (C, D) or arachidonic acids (E, F) as carbon sources. The same quantities of samples were loaded per lane and the TLC plate was developed using n-hexane:ethyl acetate (95:5) (thrice) as the solvent system. Compounds were revealed using cupric sulfate. α, alpha-mycolates; M, methoxy-mycolates, K, keto-mycolates. The TLCs of this figure represent the results of three experiments.

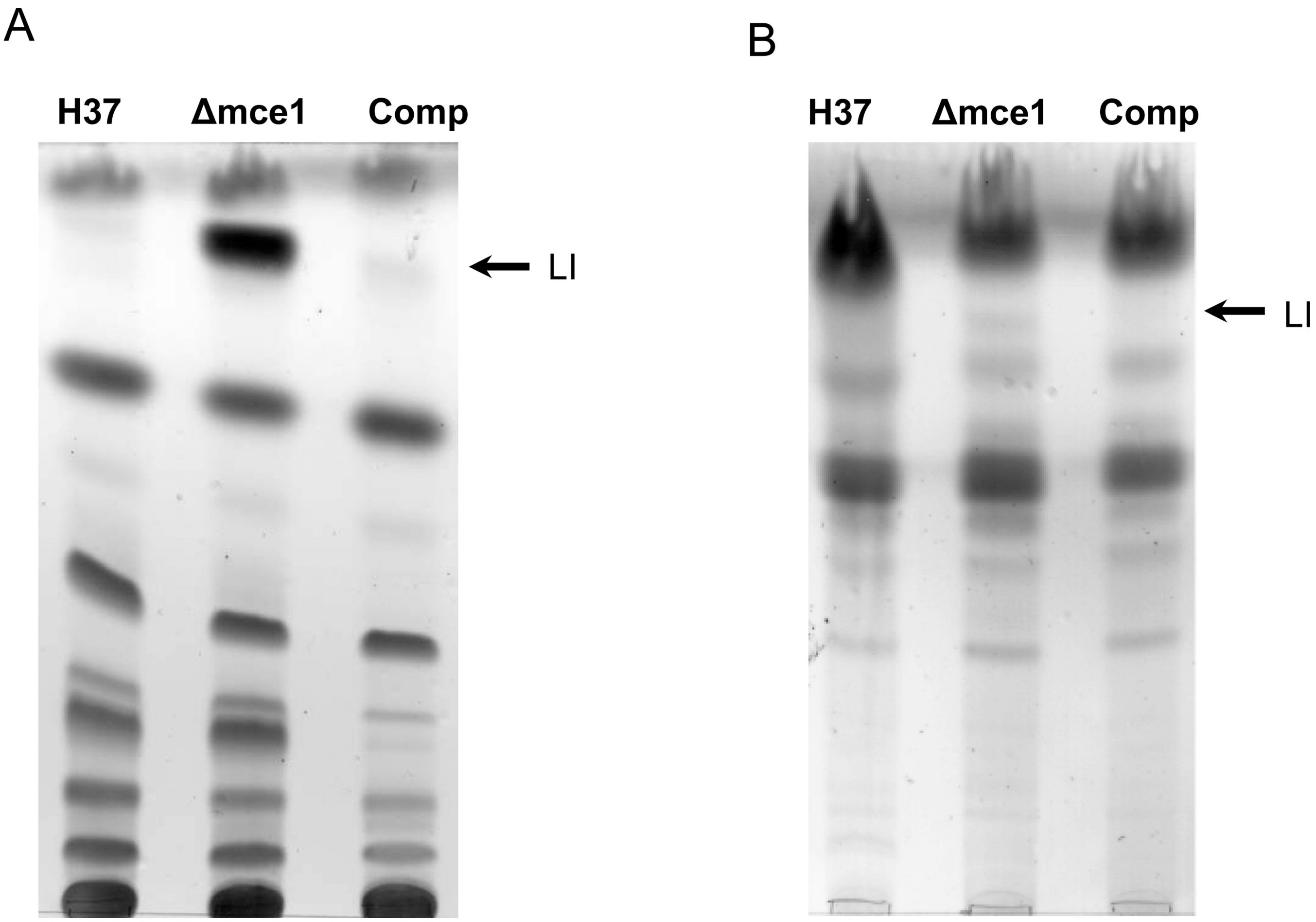


Figure 4. The lipid profile of the Δmce1 mutant is altered in response to arachidonic acid as carbon source

Cellular extractable (A) and supernatant (B) lipids from *M. tuberculosis* H37Rv (H37), the Δmce1 mutant (Δmce1) and complemented mutant strain (Comp) cultured in minimal medium supplemented with arachidonic acid. The lipids were developed by TLC from bottom to top using chloroform:methanol:water in the following proportions: 90:10:1. TLCs were stained with CuSO4. The arrows indicate the increased lipid LI in the Δmce1 mutant. These lipid patterns were repeated in three independent experiments.

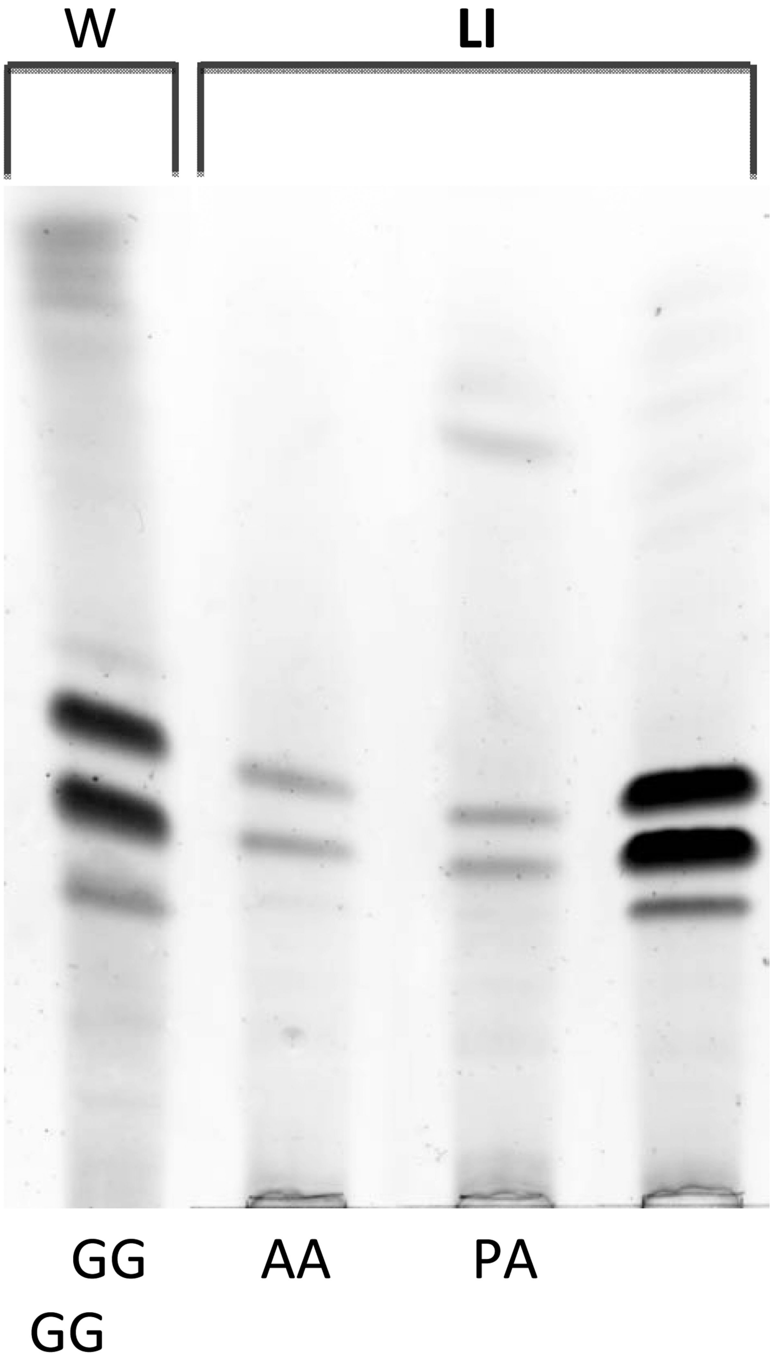


Figure 5. LI lipids are free mycolic acids

Compounds migrating at LI positions indicated in Figures 2A, 4A and Supplementary data 3A were eluted from the silica gel plate and processed as they were MAMEs/FAMEs. LI fractions obtained from Δmce1 mutant cultured in arachidonic acid (AA), palmitic acid (PA) or glucose+glycerol (GG) were developed by TLC in the solvent system nhexane: ethyl acetate (95:5) (thrice) and revealed with CuSO4 and heating. FAMEs/MAMEs prepared from whole *M. tuberculosis* cells (W) were run in parallel.

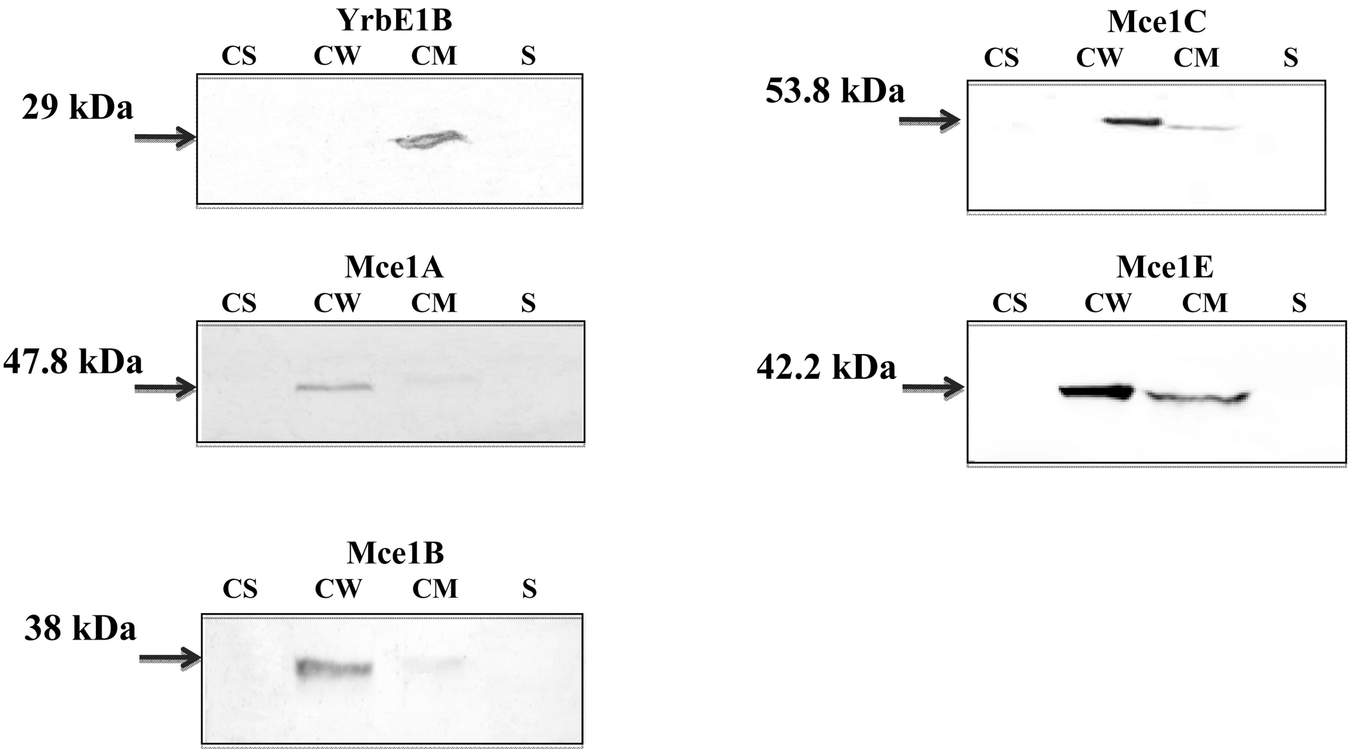

YrbE1B
CS
CW
CM
S
29 kDa
Mce1C
CS
CW
CM
S
53.8 kDa
Mce1A
CS
CW
CM
S
47.8 kDa
Mce1E
CS
CW
CM
S
42.2 kDa
Mce1B
CS
CW
CM
S
38 kDa

Figure 6. Subcellular localization of the Mce1 proteins

YrbE1B, Mce1A, Mce1B, Mce1C and Mce1E were expressed as C-ter hemaglutinine (HA) fusion proteins. The *mce* genes were cloned into the pML2031 vector, the proteins were expressed by the added of 500 µM isovalernitrilo in *M. smegmatis*. The proteins secreted to the supernatant (CS), those of the cell wall-enriched fraction (CW), membranes-enriched fraction (CM) and cytoplasmic soluble proteins (S) were resolved in a 12% SDS-PAGE and detected by western blotting using an anti-HA and anti-IgG conjugated with phosphatase as primary and secondary antibodies, respectively. Molecular weights of recombinant proteins (kDa) are indicated on the left.