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ORIGINAL ARTICLE

# Metabolic profile of *Mycobacterium smegmatis* reveals Mce4 proteins are relevant for cell wall lipid homeostasis

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**Abstract**
*Introduction* The Mce proteins are encoded in a variable number of operons (from one to eight) in all *Mycobacterium* species. A role in the transport of host and mycobacterial lipids has been demonstrated for some Mce proteins in the pathogen *Mycobacterium tuberculosis* but little is known about these proteins in *Mycobacterium smegmatis*, a soil dweller species.
*Objective* To investigate the role of Mce proteins in *M. smegmatis*.
*Method* We used a non-targeted GC–MS approach to define the metabolic profiles of knockout mutants in *mce* operons of *M. smegmatis*. Metabolomic analysis was complemented with thin layer chromatography and transcriptional studies.
*Result* We demonstrated that the lack of Mce4 proteins alters the primary carbon metabolism of *M. smegmatis* by reducing the content of fatty acids and increasing the accumulation of two stress-related compounds, glutamate/glutamine and triacyl glycerol (TAG). We also provide evidence supporting that the accumulation of TAG in a Δ*mce4* mutant depends on the bacterial redox state.
*Conclusion* These results, together with the finding that the cell surface of the Δ*mce4* mutant is significantly altered, support a role for Mce4 in maintaining the cell wall lipids architecture of *M. smegmatis*, which, when altered, induces a redox imbalance that results in the accumulation of the stress-related compounds.

**Keywords** *Mycobacterium smegmatis* · *mce4* operon · Triacyl glycerol · Glutamate

## 1 Introduction

The Mce proteins are a group of secreted or cell wall proteins encoded in large operons. Mammalian cell entry (Mce) proteins were first described to be related to bacterial entry into mammalian cells (Arruda et al. 1993). Each of the reported Mce operons contains eight genes and is organized in an identical manner: two *yrbE* genes (A and B) followed by six *mce* genes (A, B, C, D, E and F).

Operons with identical structure were identified in all *Mycobacterium* species examined, as well as in five other Actinomycetales genera. Casali and collaborators (2001) have classified the operons as *mce1-8* according to the

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clustering observed. The number of *mce* operons varies among *Mycobacterium* species: for instance, *M. tuberculosis* contains four *mce* loci (*mce1*, *mce2*, *mce3* and *mce4*), *M. bovis* three (*mce1*, *mce2* and *mce4*), *M. smegmatis* six (*mce1, 3, 4, 5, 5*bis and *7*) and *M. leprae* only has the *mce1* operon. In fact, *mce1* is the sole operon that is conserved in all the *Mycobacterium* species examined. Mce proteins are homologous to ATP binding cassette transporters (ABC-transporters), presenting a 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 (Casali and Riley 2007; Dassa and Schneider 2001). Based on bioinformatics predictions, Song et al. (Song et al. 2008) have proposed that the *mce* genes of *M. tuberculosis* encode outer membrane proteins (OMPs) which form an outer membrane complex. The energy needed to fuel substrate translocation is provided by a common ATPase, MceG, encoded in a different locus (Joshi et al. 2006).

Although the function of the Mce protein family has not been clearly established, it has been hypothesized that they are membrane transporters of lipids. Indeed, genetic and biochemical evidence support a role for *mce1* and *mce4* in the transport of free-mycolic acids and cholesterol, respectively, in *M. tuberculosis* (Forrellad et al. 2014; Pandey and Sassetti 2008). The role of *mce4* operon in cholesterol transport appears to be conserved in *M. smegmatis* and the uptake of this steroid seems to be an exclusive function of *mce4* among the six *mce* operons (Klepp et al. 2012). The redundancy of *mce* genes in actinomycetales genomes (Casali and Riley 2007), together with the fact that the mutation of *mce* genes alters the replication of pathogenic bacteria in mouse models (Sassetti and Rubin 2003; Gioffre et al. 2005; Joshi et al. 2006) and in vitro controlled conditions (Beste et al. 2009), suggest that they probably fulfil important functions in these organisms. Although substantial advances have been made in the elucidation of the function of the Mce family proteins, many questions still remain to be answered: for instance, why are the Mce proteins conserved in *M. smegmatis* (Haile et al. 2002), a soil dweller, yet they are critical for virulence in *M. tuberculosis*? They may play an identical and fundamental role in both organisms, but over the evolution of intracellular mycobacterial species this function would have been adapted to a parasitic lifestyle. Previous evidence from our group indicated that Mce proteins are implicated in the preservation of the surface properties of *M. smegmatis*, likely maintaining the homeostasis of undefined cell wall lipids (Klepp et al. 2012). In addition, we have demonstrated that Mce4 proteins are involved in cholesterol transport in *M. smegmatis* (Klepp et al. 2012). However, in spite of these evidences, not obvious alterations of the total lipid content have been detected in the absence of Mce proteins, even in the presence of cholesterol or other steroids as the sole carbon source (Klepp et al. 2012). From these results we hypothesized that the lack of Mce proteins alters the distribution of certain lipids in the bacterial cell wall without affecting the total lipid abundance. Since there are not efficient methodologies to analyse pure cell wall components of mycobacterial species, a metabolic profiling of *M. smegmatis mce* mutant strains was performed as an alternative strategy. The rationale of this approach was that changes in the metabolism of the mutant strains would shed light on the role of Mce proteins in maintaining the cell wall lipid homeostasis.

## 2 Materials and methods

### 2.1 Bacterial strains and culture media

All cloning steps were performed in *Escherichia coli* DH5α. *E. coli* were grown either in Luria–Bertani (LB) broth or on LB agar. *M. smegmatis* strains were grown in Middlebrook 7H9 medium supplemented with albumin 0.5 %, dextrose 0.4 %, and glycerol 0.5 % (M7H9-AD-G) and Tween 80 0.05 % or Middlebrook 7H11, supplemented with albumin, dextrose and glycerol. When necessary, 20 μg/mL kanamycin was added to the media. For metabolic profiling, *M. smegmatis* strains were grown either on Middlebrook 7H9 supplemented with 0.5 % glycerol, 0.4 % glucose and 0.05 % Tween 80 (M7H9-AD-G-T) or minimum medium (Sauton) supplemented with $SO_4(NH4)_2$ and 0.5 % glycerol or 0.4 % glucose as carbon sources. Oxidative or reducing stresses were generated by the addition of 5 mM of $H_2O_2$ or 1,4-dithiothreitol (DTT) to the cultures grown to late exponential phase, and collected after overnight induction for lipid analysis.

### 2.2 Complementation of the Δ*mce4* strain

Two overlapping DNA fragments (5754 bp and 4349 bp, respectively) encompassing the *mce4* operon from *M. smegmatis* and its promoter region, were PCR amplified with oligonucleotide primers (Online Resource 1) and cloned into the integrative plasmid pYUB178 to produce plasmid pYUB178 mce4. After confirming the identity of the cloned fragment by DNA sequencing and restriction enzymes (data not shown), the recombinant plasmid was used to transform *M. smegmatis* Δ*mce4* strain by electroporation. The resulting complemented strain was referred to as Δ*mce4*::*mce4*.

### 2.3 Sample preparation, metabolite extraction and derivatization

*M. smegmatis* strains were grown in either minimum media (Sauton medium supplemented with glucose or glycerol) or rich medium (M7H9-AD-G-T) until late exponential phase. Bacteria pellets were normalized by wet weight and metabolically quenched by immersion into acetonitrile:methanol:$H_2O$ (40:40:20) at 0.1 g/mL precooled to −15 to −20 °C. Metabolites were extracted by mechanical lysis using a FastPrep bead beater per quintuplicate. For derivatization, extracts were dried using a SpeedVac vacuum concentrator, and samples were methoximated by adding 50 µL of a 15 mg/mL solution of methoxyamine-HCl in pyridine, incubating for 45 s at 60 °C, sonicating for 10 s, then incubating for another 45 s at 60 °C. Following methoximation, samples were silylated by adding 50 µL of *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) +1 % trimethylchlorosilane (TMCS) incubating at 60 °C for 30 s. Following silylation, samples were centrifuged for 5 s at 4 °C, and 80 µL of the supernatant was transferred to a 150 µL glass insert in a 1.0-mL glass autosampler vial (Lisec et al. 2006).

### 2.4 Metabolite detection using gas chromatography coupled to mass spectrometry

Each sample was run in duplicate in discrete randomized blocks. A total of 1 µL of each sample was injected into the inlet and split with a 1:10 split ratio. The GC–MS settings were as follows: GC: Thermo Trace GC Ultra; MS: Thermo DSQ II; column: 30 m TG-5MS (Thermo Scientific, 0.25 mm i.d., 0.25 µm film thickness); program: 80 °C for 0.5 min, then 15 °C per min to 330 °C and held for 8 min; MS scan: 50–650 m/z at 5 scans/s; other specifications were as follows: inlet at 280 °C; auxiliary line at 300 °C; helium carrier gas flow at 1.2 µL/min; source temp: 260 °C.

### 2.5 Data processing and statistical analysis of metabolites

Raw data files (Thermo Scientific format.raw) were converted to.cdf format, and feature detection, and mass and retention time alignment was performed using the XCMS package in R.

Raw peak areas were normalized to total ion signal in R. Metabolite abundances were quantified as the mean of the chromatographic peak area for duplicate injections. Two-way analysis of variance (ANOVA) was conducted on relative metabolite abundances in R using aov function with media and strain as the main effects. ANOVA *p* values were adjusted via Benjamini-Hochberg using the p.adjust function in R. PCA was performed in R using the pca Methods package. *t* tests were performed in Excel to compare 2 groups (wild type, WT vs. Δmce4-Δ6x). GC–MS spectra were screened in both (1) National Institute of Standards & Technology library and (2) Golm Metabolome Database to assign putative identifications to molecular features. *Z* scores were calculated for each metabolite based on the mean and standard deviation of WT within each media type.

### 2.6 Lipid analysis

Bacterial were grown in the same conditions as for metabolic profiling. Since in the absence of detergents the mutants grew aggregated, the cultures without detergent were collected at the time point at which the cultures with detergent reached late exponential phase (5–6 days). Total lipids from bacterial cells and culture filtrate were extracted following procedures described earlier (Stadthagen et al. 2005). Briefly, cultures were grown to late exponential phase and total cell wall lipids extracted with chlorophorm: methanol and analysed by TLC on silica gel 60F254 loading the same lipid quantities per lane (200 µg). TLCs were developed using n-hexane: diethyl ether: acetic acid 75:25:1 solvent system to resolve TAG and revealed by spraying with a $CuSO_4$-phosphoric acid solution and heating. The intensity of the spots was quantified using ImageJ software. Values are the mean of five independent experiments and statistical analysis was performed using an unpaired *t* test.

### 2.7 RNA preparation and RT-qPCR

DNA-free RNA was extracted from 50 mL late-exponential phase cultures of *M. smegmatis* (duplicate cultures of those used for lipid analysis) as described by Santangelo et al. (Santangelo et al. 2002). RT-qPCR reactions were performed as previously described (de la Paz Santangelo et al. 2009; Blanco et al. 2012) using DNA-free RNA (1 µg) and specific primers (Online Resource 1). Relative quantification was performed by using *sigA* as a reference gene and a subsequent analysis for statistical significance of the derived results was performed by using the Pair Wise Fixed Reallocation Randomization test (Pfaffl et al. 2002). The mean value of PCR efficiency for the primers was calculated using both the classical dilution curve and slope calculation ($E = 10\ [-1/\text{slope}] - 1$) and an estimation by absolute fluorescence increase (Ramakers et al. 2003). In all cases, the expression levels were relative to the expression of the housekeeping gene *sigA*.

### 2.8 Electron microscopy

Briefly, 5-mL culture aliquotes of different *M. smegmatis* strains were concentrated by centrifugation (5000*g*), washed three times with phosphate buffer (pH 7.0), fixed for 8 h with 2.5 % glutaraldehyde in Millonig's phosphate buffer at pH 7.0. A second fixing was performed in 1 % osmium tetroxide in phosphate buffer for 1 h at 4 °C. A series of sequential ethanol dehydrations were performed for 10 min each from 50 % up to 90 % before observing the samples on a Quanta 250 FEI scanning electron microscope.

## 3 Results and discussion

### 3.1 Metabolic profiling of *M. smegmatis* Δ*mce* mutants

In order to evaluate how intermediates of central carbon metabolism and of the biosynthesis of major lipids were altered in *M. smegmatis* in the absence of the *mce* operons, we analyzed the metabolic profile of two *M. smegmatis mce* mutants using a non-targeted gas chromatography–mass spectrometry (GC–MS) metabolomic approach routinely used to quantify free fatty acids, saccharides, amines, amino acids and small organic acids. In this study, we evaluated two mutants lacking the *mce4* (Δ*mce4*) or the six *mce* operons (Δ6X) of *M. smegmatis* that were previously constructed by our group (Klepp et al. 2012). The in vitro growth of *M. smegmatis* Δ*mce4* and Δ6X mutants was similar to that of the WT strain in the presence of detergent (Online Resource 2); however, the mutant Δ*mce4* grew aggregated in the absence of detergent, as previously described for the Δ6X mutant (Klepp et al. 2012) (Fig. 1a). In addition, the mutant Δ*mce4* exhibits alteration in colony morphology (Fig. 1b) and severe cell clumping (Fig. 1c).

To compared the metabolome of mutants to that of the WT we grew the strains in a minimal medium containing either glucose or glycerol as the sole carbon source in the presence of $SO_4(NH_4)_2$, and soluble metabolites were extracted with a mixture of acetonitrile and methanol.

The non-targeted GC–MS approach resulted in the detection of 9175 molecular features (described in methods) (Online Resource 3). Analysis of variance was performed to identify effects of media and mutant treatments. Of the 9175 molecular features, 5159 (56 %) varied for mutant treatments, 8058 (88 %) varied for carbon sources, and 4807 (52 %) varied for a mutant × media type interaction (ANOVA, Benjamini-Hochberg adjusted $p < 0.05$). Principal component analysis (PCA) was used to evaluate the media and mutation effects on the GC–MS detected metabolome, represented by the 9175 molecular features. PC1 separated WT and Δ*mce4* and Δ6X grown on glycerol from the strains grown on glucose, indicating that 77.3 % of the variation was associated with the type of media used to grow the cells. PC2 explained 7.9 % of the variation and separated WT from both Δ*mce4* and Δ6X mutant strains in the glucose and glycerol groups (Fig. 2). In the PCA, a media × mutant interaction is supported by the vertical separation of WT and mutant strains for the glycerol media, and the diagonal separation for the glucose media. The ANOVA and PCA support that media type (glycerol or glucose) was the main contributor to metabolome variations. These results indicate that the deletion of the *mce4* operon, the first one deleted in the series (Klepp et al. 2012), resulted in a unique metabolite profile differing from that in the WT; however the metabolic profile was influenced by media type.

### 3.2 Fatty acid content is altered in the *M. smegmatis* Δ*mce* mutants

Metabolite abundances in the mutant strains were *z*-transformed based on the mean and standard deviation of WT for the glucose and glycerol sample types (Fig. 3). Of these, fatty acids were found to highly vary between mutants and WT (ANOVA, $p < 0.01$). Fatty acids (palmitic acid, stearic acid and oleic acid) decreased in the mutants when either glucose or glycerol was added to the medium. Similar results were obtained in bacteria cultured in rich medium (Online Resource 4). This reduction could be due to two possible scenarios: (a) an increase of fatty acids catabolism, or (b) rerouting of fatty acid biosynthesis into different metabolites.

Other fatty acid metabolism-related metabolites were found to vary between mutants and WT strains. Fatty acids are mainly catabolised by β-oxidation and the resulting acetyl-coenzyme (acetyl-CoA) is processed by the tricarboxylic acid cycle (TCA) cycle (Fig. 4). Citric acid and succinate are key components of the TCA cycle, and both were diminished in the mutants on glycerol medium compared to the WT, but the opposite was detected in glucose medium. Acetyl-CoA or propionyl-CoA, the final products of β-oxidation, were not detected in our analysis. Therefore, from these results we are not able to define whether or not the decrease in fatty acid content in the mutants was due to increase in lipid catabolism. The other possibility could be attributed to an increase of the synthesis of both the major storage compound triacylglycerol (TAG) (Garton et al. 2008) and/or the major components of the mycobacterial cell wall, such as mycolic acids and multimethyl-branched lipids. TAG cannot be detected by GC–MS, but the precursor glycerol-3-phosphate was slightly increased in the mutant strains on glucose media (Fig. 3).

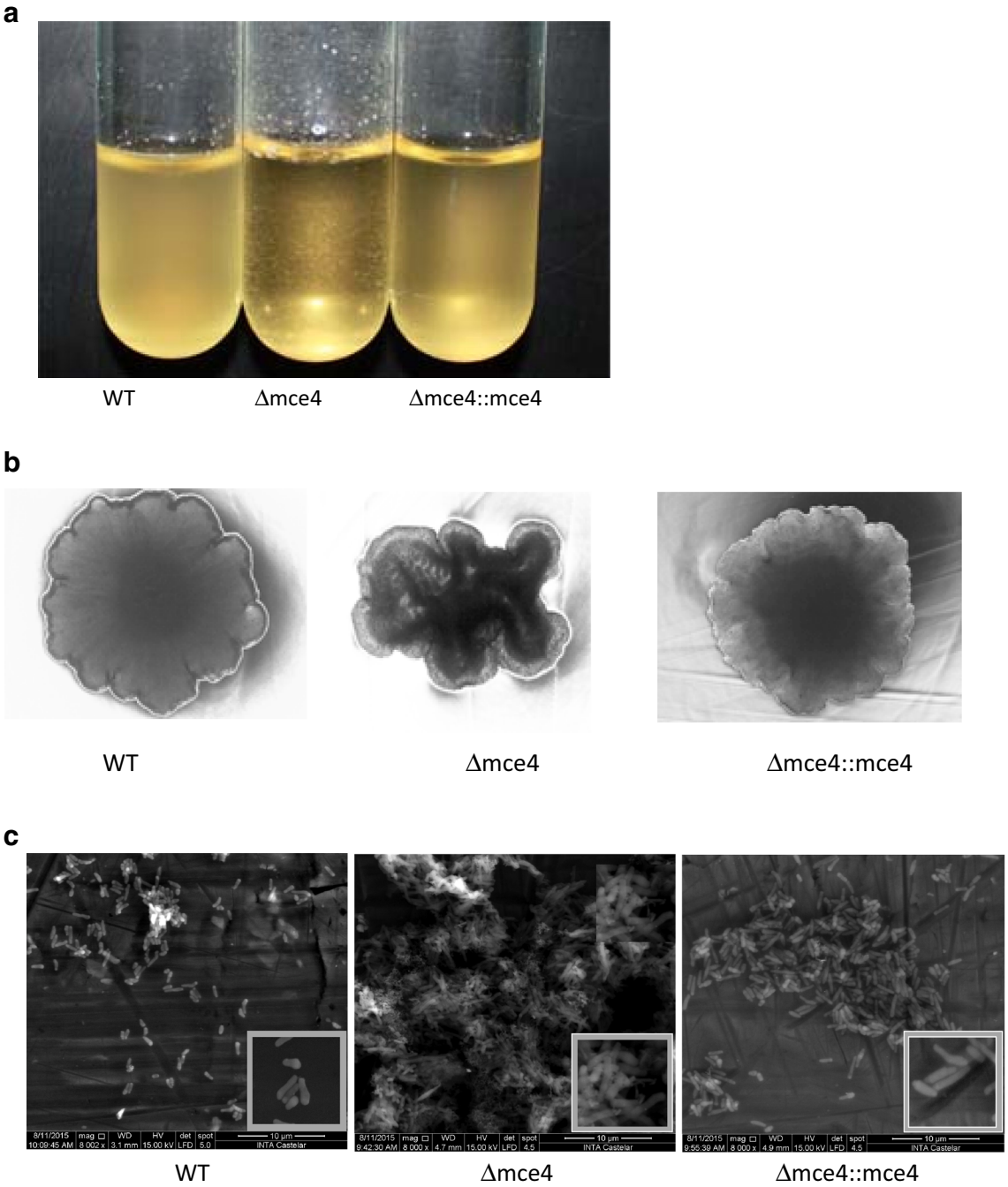


**Fig. 1** *M. smegmatis* WT, Δ*mce4* and Δ*mce4*::*mce4* strains were grown on Luria–Bertani (**a**) or Luria–Bertani agar (**b**) media during 48 h. The mutant strain displayed significant clumping and altered colony morphology as compared to the WT and complemented strains. **c** SEM analysis illustrates the severe clumping of Δ*mce4* cells. Inset; high magnification image

### 3.3 Lipid analysis of *M. smegmatis* Δ*mce4* mutant

Since the deletion of *mce4* affected the metabolic profile of *M. smegmatis* at the same extent as the deletion of all six *mce* operons, we performed further analysis only in the Δ*mce4* mutant, and we constructed the complemented strain Δ*mce4::mce4*. We investigated the effect of the deletion of the *mce4* operon on lipid metabolism. Therefore, we looked for lipid variations in the total cells and culture filtrate by thin layer chromatography (TLC) analysis. Although in the presence of glycerol as carbon source all strains produced high levels of cellular TAG, as glycerol is a critical precursor of TAG synthesis, the mutant Δ*mce4* showed a moderate increase of this lipid when

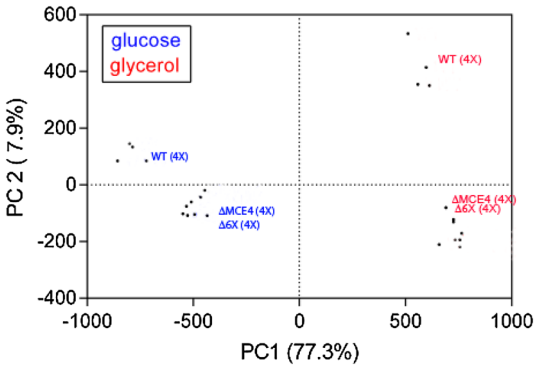


**Fig. 2** Principal component analysis. PC scores plot of the entire dataset. Δ*mce4* and Δ6x cluster together, indicating a similar metabolite profile and separated from the WT. PC1 explained 77.28 % of the variation due to the media, while PC2 explains 7.89 % due to the biotype

compared to the WT and complemented strains (Fig. 5a). When glucose was used as the carbon source, the mutant strain consistently accumulated TAG, and the wild type levels of TAG were restored in the complemented strain (Fig. 5a). These phenotypes were reproducible throughout five independent experiments. Quantification of TAG band intensities indicates that in the glucose supplemented medium the TAG content in the *mce4* mutant strain was significantly higher than and the wild and complemented strains (Fig. 5b). TAG were poorly detected in culture filtrates; however, the content was similar in all strains assayed (Online Resource 5). We also analysed the mycolic acid content in the extractable lipids from the total cells and in the culture filtrate. There were no differences in the mycolic acid profiles as assayed by TLC (data not shown).

### 3.4 Transcriptional analysis of the enzymes involved in lipid metabolism

To validate the above results, we quantified transcript abundances using qRT-PCR of genes encoding key enzymes involved in lipid metabolism. We selected isocitrate lyase (*icl*), involved in assimilating acetyl-CoA and propionyl-CoA from fatty acid catabolism through glyoxylate shunt; the TAG synthase encoded by *tgs1*, responsible for most of the TAG synthesis in vitro (Daniel et al. 2004; Sirakova et al. 2006); and four genes involved in different stages of mycolic acid synthesis, *fasI*, *acp*, *kasA* and *fabG* (Shi et al. 2010) (Fig. 4). Because the difference in the accumulation of TAG in the mutant strain compared to the WT was most notable in the presence of glucose, the enzyme expression analysis was done in bacteria cultivated in the presence of this carbon source. In line with the accumulation of TAG in the mutant strains, an up-regulation of *tgs1* in the mutant strains relative to the WT parent strain was noted. The expression of *tgs1* in Δ*mce4* mutant was higher than in the WT strain (Fig. 6). This higher expression of the *tgs1,* which is consistent with the higher TAG content of the *mce4* mutant, indicates that the lack of Mce4 proteins redirects fatty acids to these major storage compounds. Interestingly, this gene was shown to be upregulated in non-replicating *M. tuberculosis* cells (Sirakova et al. 2006). Moreover, it has been proposed that upregulation of TAG synthesis, among other pathways, is associated with *M. tuberculosis* response to stress both in vivo and in vitro (Sirakova et al. 2006; Kim et al. 2010). Transcription analysis of the *icl* gene and that of genes involved in mycolic acid biosynthesis showed that there were no consistent differences in the expression of none of these genes compared to the WT (data not shown).

### 3.5 Amino acid biosynthesis

The metabolic profiling revealed an increase in amino acids in the mutant strains, mostly when glucose was supplemented as the carbon source. Remarkably, glutamine/glutamate/pyroglutamate metabolites accumulated in the mutant strains in minimal medium supplemented with either glucose or glycerol as carbon sources (Fig. 3) and in rich medium supplemented with glycerol (Online Resource 4). This result suggests that *M. smegmatis* directs the carbon flux from α-ketoglutarate towards glutamate in the absence of Mce4 proteins (Fig. 4). This glutamate accumulation may serve as a nitrogen reserve when bacteria are under stress conditions, as previously reported (Behrends et al. 2012; Tripathi et al. 2013). In *M. tuberculosis*, ammonium assimilation mainly occurs via the glutamine synthetase and glutamine oxoglutarate aminotransferase pathway, whereas the GDH (glutamate dehydrogenase, encoded by *gdh*) pathway is mostly catabolic and results in glutamate degradation. Glutamine synthetase activity, encoded by the essential gene *glnA1* (Tullius et al. 2003; Harth et al. 2005), results in glutamine synthesis via ATP-dependent ammonium condensation with glutamate, whereas the glutamine oxoglutarate aminotransferase pathway, catalyzed by glutamate synthase [NADPH, large subunit] (encoded by *gltB*) synthesizes glutamate from glutamine and α-ketoglutarate (Gouzy et al. 2014). Therefore, we looked at the transcriptional variations for two genes involved in nitrogen metabolism (Fig. 4). There were no consistent differences in the expression of both enzymes among strains (Fig. 6). This suggests a post-transcriptional mode of regulation for glutamine/glutamate. However, it should be noted that the GC–MS metabolomics method employed for this study uses a derivatization technique that can be confounded by spontaneous conversion of glutamine to both glutamate and pyroglutamate, and so the three metabolites are difficult to distinguish.

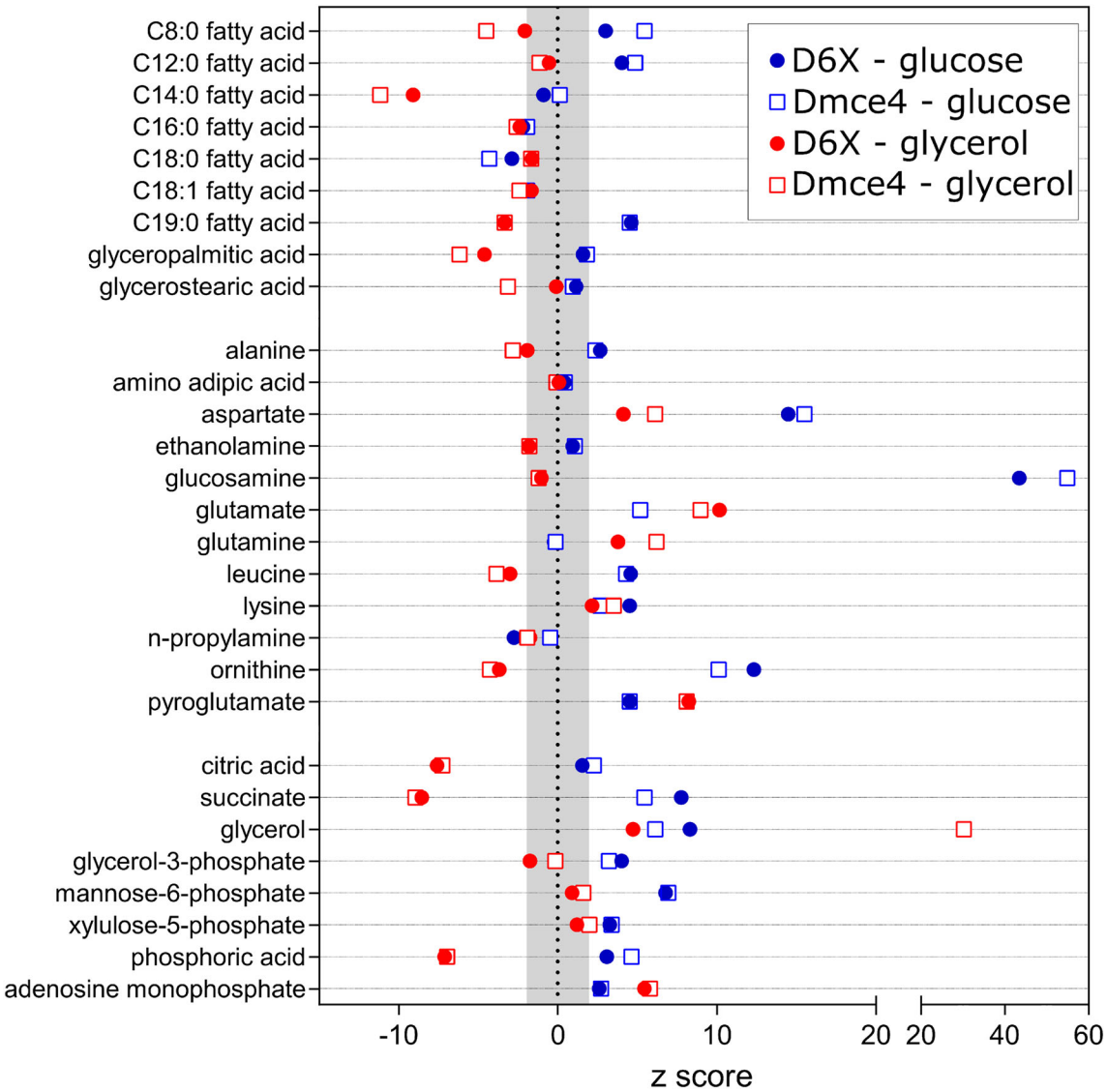


**Fig. 3** Metabolic profile of the *M. smegmatis* WT and mutant strains (Δ*mce4* and Δ6X) grown on minimum media Sauton supplemented with $SO_4(NH_4)_2$, and glucose or glycerol as carbon sources. Samples were run in a GC Thermo Scientific iSQ-TRACE DSQII. GC–MS spectra were screened in both (1) National Institute of Standards & Technology library and (2) Golm Metabolome Database to assign putative identifications to molecular features. Results are shown as a *z*-score plot

### 3.6 Evaluation of TAG accumulation under redox stress conditions

Collectively, the above results showed that the carbon flow of the mutant strain is rerouted towards the accumulation of TAG and glutamate. Although, mycobacteria produce TAG and glutamate in normal physiological conditions, several examples in the literature have demonstrated that these molecules are accumulated when mycobacteria are exposed to various stress conditions. While Sing et al. have proposed that intracellular oxidative or reductive stress in *M. tuberculosis* modulate anabolism of TAG and diverse polyketides (Singh et al. 2009), Gallant et al. have shown that *Mycobacterium bovis* BCG uses glutamate as a carbon source and as protectant against acidic and nitrosative stresses (Gallant et al. 2016). Moreover, Cowley et al. (Cowley et al. 2004) have suggested that an increase in glutamate and glutamine levels may provide a pool of amines for synthesis of macromolecules, such as mycothiol, required for *M. tuberculosis* to deal with oxidative stress. Based on the proposed role of Mce proteins as transporter systems of compounds from outside to inside, it is likely that the substrates of Mce4 transporter are the messengers of these stress signals. In a previous study, we demonstrated that Mce4 participates together with other transporters in the uptake of cholesterol in *M. smegmatis*,

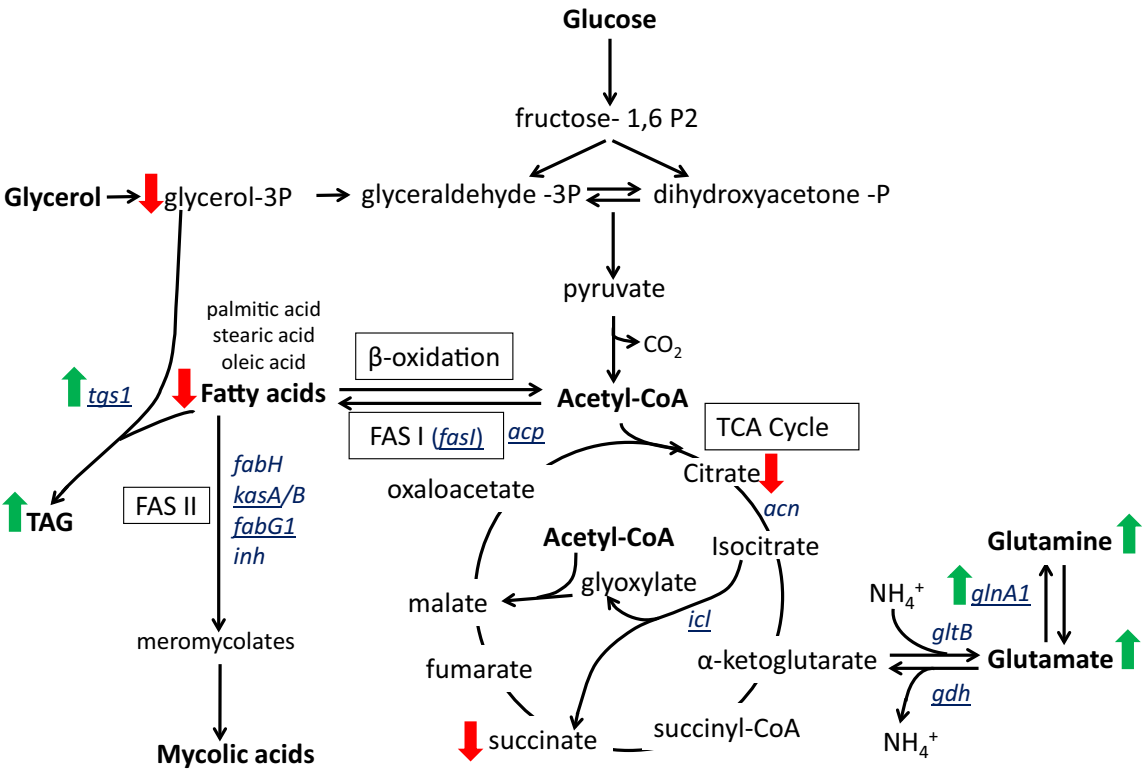


**Fig. 4** Pathways involved in central metabolism and lipid metabolism in *Mycobacterium* sp. are schematized. *Green* and *red arrows* indicate increase and decrease of the compound/transcript in the mutant compared to the WT strain, respectively. Genes encoding key enzymes involved in lipid metabolism selected for qRT-PCR analysis are underlined in the figure. Glyoxylate shunt: *icl* (Rv0467, MSMEG_0911), isocitrate lyase; glutamate and glutamine synthesis: *glt*B (Rv3859, MSMEG_3225), glutamate synthase (large subunit); *gln*A1 (Rv2220, MSMEG_4290) glutamine synthetase; *gdh* (Rv2476c, MSMEG_4699) glutamate dehydrogenase; TAG synthesis: *tgs*1 (Rv3130c, MSMEG_3948), TAG synthase; Mycolic acid synthesis: *fas* (Rv2524c, MSMEG_4757), fatty acid synthase; *acp* MSMEG_1490 3-oxoacyl-ACP synthase III¸ *fab*H (Rv0533c), β-ketoacyl-AcpM synthase III; *kas*A(Rv2245, MSMEG_4327) β-ketoacyl-AcpM synthase; *fab*G1 (Rv1483, MSMEG_3150) β-ketoacyl-AcpM reductase

as reported in *M. tuberculosis*. However, cholesterol is not a frequent carbon source in the *M. smegmatis* environments and, in this study, the accumulation of TAG in the absence of Mce4 proteins was detected in a cholesterol-free medium. On the other hand, our group and others have proposed that Mce1 proteins participate in the mycolic acid recycling of *M. tuberculosis* cell wall (Forrellad et al. 2014; Dunphy et al. 2010; Cantrell et al. 2013). Based on the high homology among *mce* operon sequences, we propose that the lack of Mce4 proteins may abrogate the recycling of certain undefined lipids, and thus, the structural integrity of the cell wall structure is altered. This change in the architecture of the cell wall lipids would trigger, among other alterations, a redox stress response that results in the accumulation of TAG and glutamate. Therefore, we hypothesized that the absence of Mce4 produces a reductive environment that induces compensatory changes, such as accumulation of TAG. Under this model, we reasoned that the external modification of this reductive environment should revert this TAG accumulation. To contrast this hypothesis, we analysed the accumulation of TAG under oxidizing and reducing conditions. To this end, cells were exposed to 5 mM $H_2O_2$ (oxidative conditions) or DTT (reductive conditions) and TAG accumulation was analysed under these conditions. As shown in Fig. 5c, while in the presence of DTT TAG were evenly produced in the mutant and in the WT, in the presence of hydrogen peroxide the amount of TAG decreased in the mutant strain as compared to the WT and complemented strains. These results are consistent with our model where changes in the mycobacterial cell wall structure, due to the lack of the Mce4-depending lipid-recycling, produces a reductive intracellular environment that contributes to the production of TAG. Supporting these findings, a *M. tuberculosis* mutant for the *whiB3* gene showed an aggregated phenotype and accumulated TAG, phthiocerol dimycocerosate and methyl-branched lipids (Singh et al. 2009). The authors demonstrated that WhiB3 modulates the synthesis of cell wall lipids in response to oxido-reductive stress to maintain redox balance.

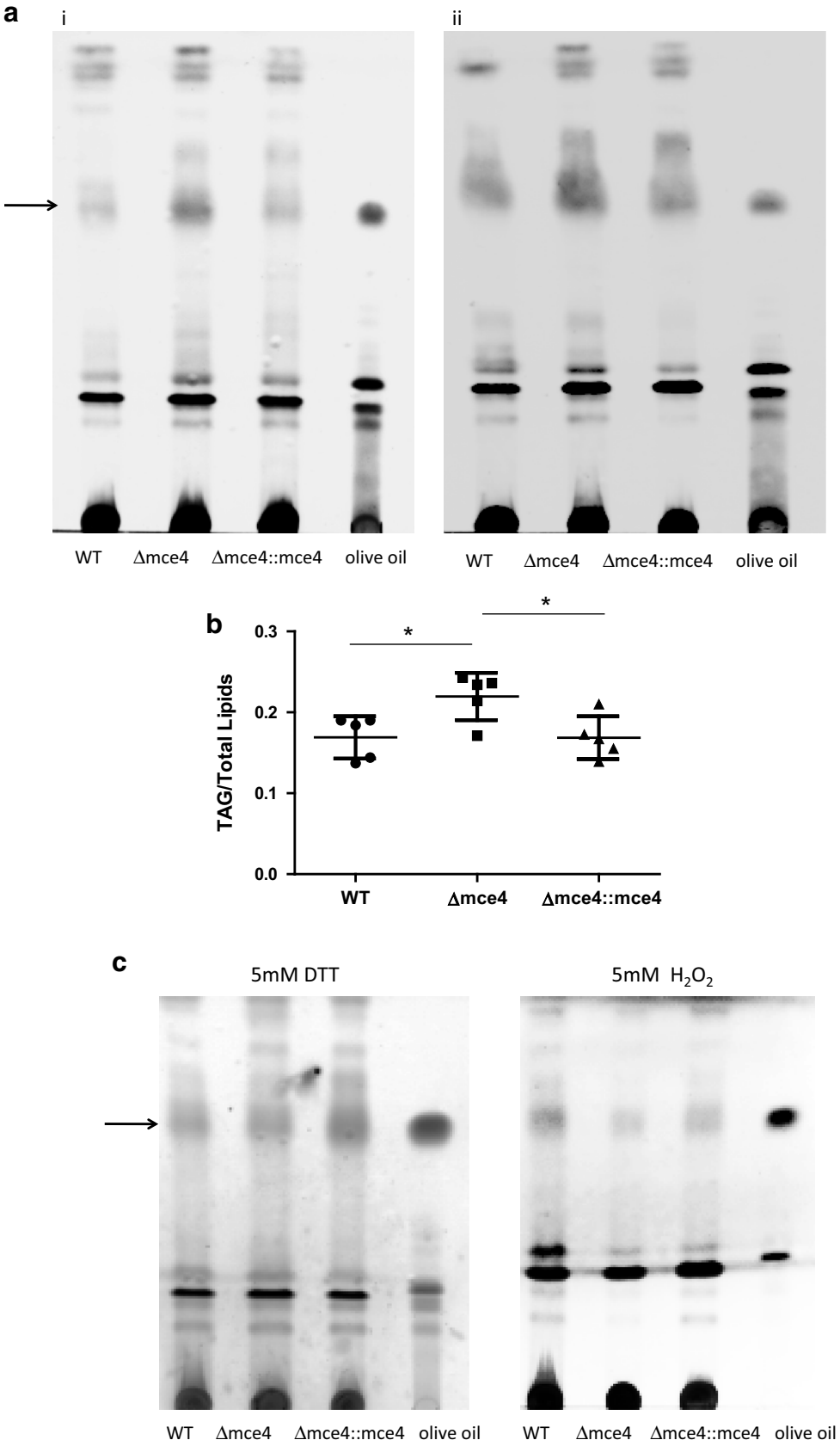


**Fig. 5** Production of TAG in *M. smegmatis* strains. **a** *M. smegmatis* strains were grown on Sauton medium supplemented with $SO_4(NH_4)_2$ and glucose (*i*) or glycerol (*ii*) as carbon source. Total lipids from cells were extracted and analyzed on TLC. Equivalent amounts of total lipids were loaded in each lane. TAG were resolved with the Solvent system *n*-hexane:diethylether:acetic acid 75:25:1. Olive oil was run as TAG control. *Arrows* indicate the position of TAG. **b** The accumulation of TAG relative to total lipids was determined in each strain by quantification of band intensities in scanned TLC. Significance was determined by unpaired *t* test, statistically significant $p < 0.05$ (*asterisk*). **c** *M. smegmatis* strains were grown at a late exponential phase as indicated in **a** and redox stresses were induced by addition of 5 mM of $H_2O_2$ or DTT and cultivated for 24 h. The **a** shows a representative experiment of five experiments while the **c** shows a representative experiment of three experiments

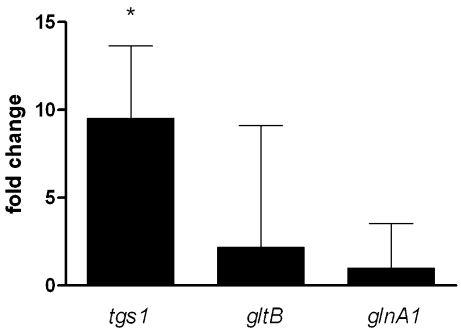


**Fig. 6** Gene expression ratios of selected genes. The *bars* indicate the average ratios of mutant/WT for three independent biological replicates, and the error bars indicate the standard deviations. The value of *tgs1* expression in the mutant was significantly different of that of WT strain, as determined by Pair Wise Fixed Reallocation Randomisation Test software

## 4 Concluding remarks

In this study, we found that the absence of Mce4 proteins significantly alters the metabolic profile of *M. smegmatis*. The *mce4* mutant accumulates less free fatty acids and greater amounts of glutamine/glutamate and TAG than the WT strain. Collectively, the results of the present study showed that the carbon flow of the mutant strain is rerouted towards the formation of storage or stress compounds, such as TAG and glutamate. We also confirmed a role of the TAG in the mechanism of intracellular redox homeostasis and demonstrated that the lack of Mce4 proteins increases the accumulation of reducing equivalents, likely due to alteration of the normal cell wall lipids recycling. These results together with the fact that the cell wall is altered in the mutant strain provide strong evidence supporting that the lack of Mce4 proteins abrogates the recycling of certain cell wall undefined lipids that triggers a redox stress response.

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**Compliance with ethical standards**

**Conflict of interest** María Paz, Santangelo; Adam, Heuberger; Federico, Blanco; Marina, Forrellad; Catalina, Taibo; Laura, Klepp; Julia, Sabio García; Pablo I., Nikel; Mary, Jackson and Fabiana, Bigi declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants or animals performed by any of the authors.

**Informed consent** Informed consent was not requested because this article does not contain studies with human participants performed by any of the authors.

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