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Deletion of MSMEG_1350 in *Mycobacterium smegmatis* causes loss of epoxy-mycolic acids, fitness alteration at low temperature and resistance to a set of mycobacteriophages

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Abstract

Mycobacterium smegmatis is intrinsically resistant to thiacetazone, an anti-tubercular thiourea; however we report here that it causes a mild inhibition in growth in liquid medium. Since mycolic acid biosynthesis was affected, we cloned and expressed *Mycobacterium smegmatis* mycolic acid methyltransferases, postulated as targets for thiacetazone in other mycobacterial species. During this analysis we identified MSMEG_1350 as the methyltransferase involved in epoxy mycolic acid synthesis since its deletion led to their total loss. Phenotypic characterization of the mutant strain showed colony morphology alterations at all temperatures, reduced growth and a slightly increased susceptibility to SDS, lipophilic and large hydrophilic drugs at 20 °C with little effect at 37 °C. No changes were detected between parental and mutant strains in biofilm formation, sliding motility or sedimentation rate. Intriguingly, we found that several mycobacteriophages severely decreased their ability to form plaques in the mutant strain. Taken together our results prove that, in spite of being a minor component of the mycolic acid pool, epoxy-mycolates are required for a proper assembly and functioning of the cell envelope. Further studies are warranted to decipher the role of epoxy-mycolates in the *M. smegmatis* cell envelope.

INTRODUCTION

Members of the genus *Mycobacterium* display one of the most intricate metabolisms of fatty acids and lipids described so far in bacteria. The synthesis of the very long α-alkyl β-hydroxy fatty acids, known as mycolic acids, is one of the most important and complex metabolisms. All mycobacterial species synthesize the core mycolate structure through conserved mechanisms: however, the core modifications are different depending on the species [1]. It has been shown that pathogenic slow growing mycobacteria such as *M. tuberculosis* and *M. bovis* produce significant amounts of α-mycolic acids with two *cis* cyclopropane rings on the meromycolate chain or oxygenated mycolates containing either a distal methoxy or keto group and a proximal *cis* or *trans* cyclopropane ring, thus yielding an extraordinary array of subtle chemical differences that has a great impact on the mycobacterial-host cross talk [2, 3]. The cyclopropane rings, keto, methoxy and methyl branches of these lipids are synthesized by a family of S-adenosyl methionine-dependent methyltransferases (designated as mycolic acid methyl transferases, MAMTs), which are highly similar in both primary sequence and tertiary structure, thus making it difficult to assign functions to each of them [4]. However, through gene knock-out, their precise roles in the mycolic acid synthesis in *M. tuberculosis* were finally deciphered [3, 5, 6]. Those studies also led to the evaluation of the impact of the loss of different families and sub-families of mycolic acids in *M. tuberculosis* and *M. bovis* BCG strain Pasteur [5–9]; two of those reports described the genes encoding the proteins responsible for the cyclopropanation of the distal and proximal double bonds of diunsaturated mycolic acids in *M. tuberculosis* [7, 9]. Later on, it was elegantly demonstrated that loss of all mycolic acid modifications (namely cyclopropanation, keto and methoxy groups)

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Keywords: *Mycobacterium smegmatis*; epoxy-mycolic acids; cell envelope alterations; temperature adaptation; mycobacteriophages.
Abbreviations: ADS, albumin-dextrose-salt; CP, cyclopropanated mycolic acid; CV, crystal violet; ems, epoxy mycolate synthase; ERY, erythromycin; FAMEs, fatty acid ethyl esters; GLs, glycolipids; GPLs, glycopeptidolipids; INH, isoniazid; MAMEs, mycolic acid methyl esters; MAMTs, mycolic acid methyl transferases; NOV, novobiocin; RIF, rifampicin; TAC, thiacetazone; TLC, thin-layer chromatography; Tr, Triton WR1339; Tw, Tween 80; VAN, vancomycin.
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One supplementary figure is available with the online version of this article.

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rendered *M. tuberculosis* highly attenuated without affecting viability but with profound changes in colony morphology and susceptibility to environmental insults [10].

It was previously reported that thiacetazone (TAC), an anti-tubercular pro-drug activated by the mycobacterial mono-oxygenase EthA [11] exerted its effect on *Mycobacterium bovis* var. BCG through inhibition of the cyclopropanation of mycolic acids [2]. This conclusion was based on the observation of the accumulation of unsaturated mycolic acid precursors with chemical structures compatible with the inhibition of a cyclopropane mycolic acid synthase. However, we showed that TAC causes mycolic acid biosynthesis inhibition in *M. tuberculosis* H37Rv and *M. kansasii* finally demonstrating that resistance to TAC in *M. tuberculosis* is mediated by mutations in *had*ABC, the mycolic acid dehydratase complex [12].

While characterizing the intrinsic resistance of *M. smegmatis* to TAC we found that this compound caused a mild growth inhibition on liquid cultures of the parental strain *M. smegmatis* mc^2155; surprisingly, the activity of this drug in liquid cultures of this saprophytic mycobacterial species has never been addressed. Due to the involvement of *M. bovis* MAMTs as targets for TAC we set out to clone and express *M. smegmatis* MAMT genes with the goal of identifying the one(s) inhibited by that drug. We herein report that one of them, *MSMEG*_1350 encodes the sole enzyme responsible for the synthesis of the epoxy family of mycolic acids and that loss of this family affects the ability of *M. smegmatis* to grow at low temperatures as well as cell-envelope functions; supporting a role of epoxy-mycolates in the cell-envelope organization and properties in spite of being the minor component of the mycolic acid family in this species.

METHODS

Bacterial strains, culture medium and growth conditions

M. smegmatis mc^2 155 and its derivatives were cultured in Middlebrook 7H9 broth (Difco, NJ, USA) supplemented with 0.5 % (w/v) glycerol and 10 % (v/v) of ADS (Albumin 50 g w/v, Dextrose 20 g w/v, NaCl 8.5 g w/v), hereafter mentioned as 7H9-ADS-Gly. The medium culture broth was supplemented with Tween 80 (Tw, Sigma, 0.2 % w/v) or Triton WR1339 (Tr, Sigma, 0.25 % v/v) when corresponded. Solid 7H9-ADS-Gly medium was obtained after the addition of agar 1.5 % (w/v). *Escherichia coli* strain DH5α was used for cloning experiments and was grown in Luria–Bertani (LB) broth or agar medium. When necessary, kanamycin or hygromycin were added at a concentration of 20 and 50 mg ml^{-1}, respectively.

All the bacterial strains were grown at 37 °C unless indicated otherwise.

Antibiotics, drugs and chemicals were from Sigma (Saint Louis, MO, USA) unless indicated differently.

Cloning of *M. smegmatis mma* genes

The *mma* genes were amplified by PCR under standard conditions, using *M. smegmatis* chromosomal DNA as a template. The amplification products were purified from agarose gels and cloned using the TopoZeroBlunt cloning vector (InVitrogen) followed by electroporation in *E. coli*. Clones containing inserts of the expected size (judged by restriction enzyme digestion) were sequenced at a commercial facility, and inserts that showed no mutations were cloned into the shuttle *E. coli*-mycobacteria vector pMV261 [13]. The complementing *MSMEG*_1350 clone was constructed by PCR amplification using oligonucleotides 1350 Fw EcoRI (GAATTCATGTCTAAATTGACACCCAAA TACG) and 1350 Rv HindIII (AAGCTTACAAATTTAC TTCTGCAGCGTGAAC) and cloning of the resulting 861 bp fragment into the pGEM T-Easy vector (Promega, Madison, WI, USA). Afterwards, a clone free of amplification errors was cloned into pMV261. In order to have an integrative construct, the XbaI-HindIII fragment spanning *hsp*60 and the *MSMEG*_1350 *orf* was sub-cloned into pML1342 [14], yielding pML1342::*MSMEG*_1350.

The *M. tuberculosis mma*A4 gene, previously cloned in pMV261 [12], was sub-cloned into pML1342 as XbaI-HindIII fragment in order to include the *hsp*60 promoter from the original construct.

Construction of the *M. smegmatis* Δ*MSMEG*_1350 mutant

*MSMEG*_1350 was deleted using the counter-selectable marker *xylE* and a thermosensitive origin of replication in the shuttle plasmid pPR27 [15]. For the deletion of the *MSMEG*_1350 gene, upstream and downstream fragments of the gene were amplified with the primers 1350 Up Fw (CGGGTCTAGAGACCCTGCTCGATGTCGGATG), 1350 Up Rv (CCCGGAATTCACTCGTTCGAAATGTCGTAGA TC), 1350 Dn Fw (CCCGGAATTCACTCGTTCGAAATG TCGTAGATC) and 1350 Dn Rv (GCCCAAGCTTACTAG TGGCGACCTACAGGTTCGATACA). A kanamycin cassette was inserted between the two fragments, cloned in pPR27 digested with XbaI and SpeI and transformed in *M. smegmatis* mc^2155 followed by selection of knock-out mutants in plates with 10 % sucrose at 42 °C as described previously [15]. Deletion of the gene was confirmed by PCR using oligonucleotides Fw1 (GCAGACAAATTTAC TTCTGCAGC) and Rv1 (ATGTCTAAATTGACACC-CAAATA).

Determination of the MIC

The MIC 99 % (MIC_{99}) was determined in 7H9-ADS-Gly solid medium by plating 200–300 c.f.u. of the parental or mutant strain on plates containing increasing concentration of the chosen compounds – novobiocin (NOV), rifampicin (RIF), vancomycin (VAN), erythromycin (ERY), thiacetazone (TAC), crystal violet (CV) and isoniazid (INH) – followed by incubation for 3 days at 37 °C or 7 days at 20 °C. MIC_{99} was defined as the minimum concentration of drug

required to produce a 99 % reduction in c.f.u. respect to control (no drug) plates.

Macroscopic and microscopic phenotypic characterization of *M. smegmatis* Δ*MSMEG_*1350

Colony morphology was determined by plating dilutions of freshly grown stationary phase cultures of wild-type and mutant strains on 7H9-ADS-Gly agar plates. Congo Red (Sigma), an azo-dye with a well-known affinity for lipids and lipoproteins, was added to the solid medium at 100 µg ml^{-1} to improve visualization of colony morphology alterations as described by Cangelosi *et al.* [16]. After 7 days at 20 or 25 °C, 5 days at 30 °C and 3 days at 37 or 42 °C, plates were visually inspected followed by optical microscopy examination at low magnification (x2) using a Zeiss-Stemi 2000 binocular scope. Several individual colonies were examined and photographed in at least three individual experiments.

Growth in liquid medium was done by diluting saturated cultures of both parental, mutant and complemented strains grown in Middlebrook 7H9-ADS-Gly-Tr were diluted into fresh medium to an OD_{600nm} = 0.1–0.2 and grown at chosen temperatures (20, 25, 37 or 42 °C), growth was followed by OD_{600nm} at different time points. The experiment was performed in triplicate. Aliquots of each culture were subjected to microscopic observation after acid-fast and Gram staining following standard laboratory protocols.

Biofilm formation

The protocol described by Ohja *et al.* [17] was used to study the ability to form biofilms by *M. smegmatis* mc^2155 and *M. smegmatis* Δ1350. Briefly, the *M. smegmatis* parental and mutant strains were grown in modified M63 media and 10 µl of these saturated cultures were inoculated in 1 ml of modified M63 media in 24-well polystyrene culture plates [18]. Alternatively, standard Sauton's media was used [19]. The plates were wrapped in parafilm and incubated at 20, 30 or 37 °C for 7 days. After that incubation period, the biofilm mass was quantitated according to Recht *et al.* [18]. To this end, the spent medium was removed from the wells, which were washed twice with distilled water; after that, 4 ml of a solution of 1 % (w/v) crystal violet was added followed by incubation for 30 min at room temperature. Afterwards, the staining solution was removed and the wells washed three times with distilled water followed by extraction of the dye with 4 ml ethanol for 1 h. Finally, absorbance at 570 nm (Abs_{570}) was measured. Viable cells present in the initial inoculum added to each well were determined by serial dilution and plating on solid medium. Typically, experiments were performed three times in triplicate using independent cultures. The c.f.u. values obtained for parental and mutant strains were similar, thus Abs_{570} values were compared directly.

Sliding motility

The assay was performed as described by Deshayes *et al.* [20], briefly 5 µl aliquots of saturated liquid cultures of the wild-type and mutant strains were deposited in the centre of plates containing 7H9 medium plus 0.3 % agarose with no added carbon source. These plates were then sealed with parafilm and incubated at 37 °C for 1 week prior to inspection.

Sedimentation assay

Sedimentation assays were carried out as described by Jamet *et al.* [19]. Briefly, 5 ml of fresh stationary cultures grown in 7H9ADS-Gly-Tw 0.2 % were diluted until an OD_{600nm} of ~1 and left standing at 37 °C. Viable cells were counted by serial dilution and plating of aliquots (1 ml) taken from the top of the culture after 1 and 24 h of the start of the assay.

Congo Red binding assays

The Congo Red binding assay was performed as previously described by Etienne *et al.* [21] with some modifications. Cultures were grown in triplicate for 3–5 days at 20 or 37 °C under shaking in 7H9-Gly-Tw 0.05 % and Congo Red dye 100 µg ml^{-1}. Bacterial pellets were obtained after centrifugation for 10 min 3000 ***g***, resuspended in 1 ml water and sonicated for 15 min in water ultrasonic cell disruptor. Then cells were extensively washed with water until supernatant was colourless and dry weight was measured. The bacteria were resuspended in 1 ml acetone, vortexed and let stand at room temperature for 1 h before centrifugation. The Abs_{488} of Congo Red present in the supernatants was measured spectrophotometrically and ratio OD_{488}/cells dry weight (mg) was calculated.

Incorporation of [1-^{14}C] acetate into fatty and mycolic acids

M. smegmatis mc^2 155 and its derivatives were grown in Middlebrook 7H9-ADS-Gly-Tw 0.2 % at 37 °C until an OD_{600nm}= 0.5–0.6, at that point the cultures were incubated during 3 h with [1-^{14}C] acetate (sodium salt; 58,9 mCi $mmol^{-1}$; Perkin Elmer, Boston, MA, USA) at a concentration of 1 µCi ml^{-1} TAC activity on mycolic acid synthesis was analysed as follows: cultures (typically 10 ml) of *M. smegmatis* mc^2155 and its derivatives at OD_{600nm}= 0.5, were split into two identical aliquots, one of which was treated with TAC at 25, 50, 100 and 200 µg ml^{-1} and further incubated for 3 h at 37 °C. Treatment was continued by further incubation with [1-^{14}C] acetate to both control- and drug-treated cultures of each strain for 3 h. The ^{14}C-labelled cells were harvested by centrifugation, washed twice with water and used immediately or kept frozen at −20 °C until use.

Extraction and analysis of radiolabelled fatty acids

^{14}C-labelled control and treated cells were processed as described [22]. Briefly, cell pellets were resuspended in 15 % (w/v) tetrabutyl ammonium hydroxide (Fluka) and incubated at 100 °C overnight. Then 2 ml of CH_2Cl_2 and 100 µl of CH_3I was added, followed by mechanical mixing by rotation for 1 h and centrifugation. The lower organic phase was then removed, washed with water and dried. A known aliquot (50 000 cpm) of the resultant mixture of fatty acid methyl esters (FAMEs) and mycolic acid methyl esters

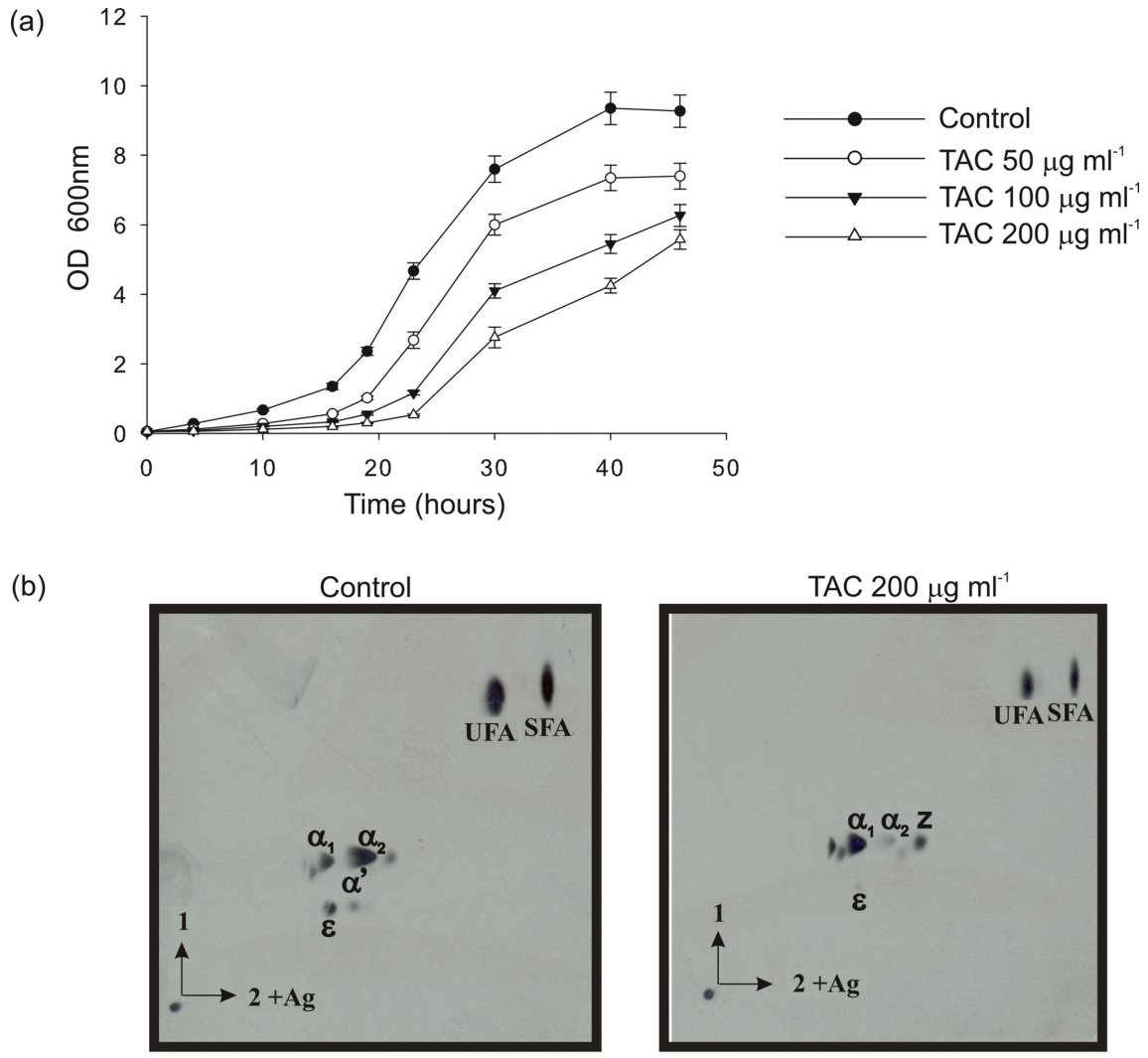

Fig. 1. Thiacetazone inhibit synthesis of α2- and ε-mycolates in *M. smegmatis* mc^{2}155. (a) Growth curves of *M. smegmatis* mc^{2}155 at 37 °C incubated in the presence of increasing concentrations of TAC. Control (no drug) (-); TAC: 50 μg ml^{-1} (™); 100 μg ml^{-1} (q) and 200 μg ml^{-1} (r). (b) 2D-TLC of 1 [^{14}C] acetic acid radiolabelled *M. smegmatis* mc^{2}155 MAMEs and FAMEs in the absence (left panel) and in the presence of TAC 200 μg ml^{-1} (right panel). Cultures were grown to OD_{600nm}= 0.5 and split into two identical aliquots, one was treated with TAC 200 μg ml^{-1} and the other was left untreated. Both cultures were further incubated for 3 h at 37 °C. Radiolabelled [1-^{14}C] acetate was added to treated cultures and cells were harvested after 3 h incubation. MAMEs and FAMEs were extracted as detailed in Methods. Aliquots containing 100 000 cpm were loaded in silver ion argentation TLC plates. Plates were developed in the first direction with hexane-ethyl acetate (95 : 5 v/v) three times and in the second direction twice with petroleum ether-diethyl ether (17 : 3 v/v). Autoradiograms were obtained after exposure to Kodak BioMax XAR film at −70 °C for 24 h. SFA, saturated fatty acids; UFA, unsaturated fatty acids.

(MAMEs) were subjected to analytical thin-layer chromatography (TLC), using silica gel plates (5735 silica gel 60 F254; Merck) with two different solvent systems: (a) in hexane-ethyl acetate (90 : 10, v/v) three times and (b) in CH_2Cl_2 once. Mono-dimensional silver ion argentation TLC was performed on silica gel plates to which aqueous $AgNO_3$ (10 % w/v) was applied, followed by drying at 100 °C for 12 h. Sample aliquots (representing ≈50 000 cpm) were applied and developed three times with a mobile phase of petroleum ether-diethyl ether (17 : 3 v/v). Alternatively, dichloromethane was used as the mobile phase for a single development. For two-dimensional silver ion argentation TLC (2D-TLC) ≈100 000 cpm of the mixture of FAMEs and MAMEs was applied to silica gel plates previously impregnated with aqueous $AgNO_3$ (10 % w/v) up to 3/4 of the height of the plate. The plates were developed in the first direction with hexane-ethyl acetate (95 : 5 v/v) three times and in the second direction twice with petroleum ether-

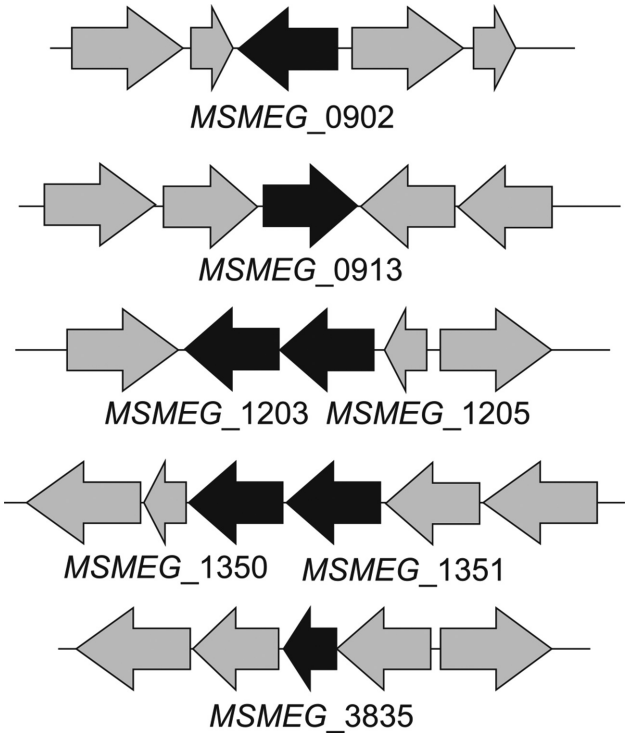

Fig. 2. Genetic organization of putative mycolic acid methyltransferase (*mma*) genes. Chromosomal loci encoding putative mycolic acid methyl transferases (*MSMEG*_0902, *MSMEG*_0913, *MSMEG*_1205, *MSMEG*_1203, *MSMEG*_1350, *MSMEG*_1351 and *MSMEG*_3835) present in *M. smegmatis* mc^{2}155 are shown.

diethyl ether (17 : 3 v/v). In all cases autoradiograms were obtained after exposure to Kodak BioMax XAR film at −70 °C for 24–48 h.

Oxidative, acidic, detergent and osmotic stress challenge experiments

Cultures of both the parental and the mutant strains were grown in 7H9-ADS-Gly-Tw medium at 37 °C until an OD_{600nm}~0.4–0.6 was reached. At that point the chosen stress agent was added to both cultures over a 3 h period and survival rate was estimated by withdrawing and plating aliquots on solid medium followed by counting the c.f.u. after incubation for 3–4 days at 37 °C. Acid stress was applied by drop-wise addition of 1M ClH to a final pH=3 as determined in preliminary experiments; oxidative stress was based on the conditions described by Li *et al.* [23], achieved by addition of H_2O_2 to a final concentration of 0.3 mM (low concentration) or 5 mM (high concentration); susceptibility to SDS as a detergent stress was done by adding concentrated (10 % w/v) SDS to a final concentration of 0.1 % (w/v) [19]; finally osmotic stress was presented by increasing the content of NaCl in the culture medium to final concentrations ranging from 0.5 to 2 M NaCl [24]. c.f.u. were determined by serial dilution plating before and after each challenge.

Bacteriophage susceptibility

The susceptibility of the mutant strain to a set of 30 mycobacteriophages belonging to different genetic groups (seven obtained from G. Hatfull and 23 isolated in our lab) [25], were used following standard protocols [26]. Briefly, 1 ml aliquots from early stationary phase cultures of both the parental and mutant strains grown in 7H9ADS-Gly-Tw 0.2 % were centrifuged 10 min at 6000 r.p.m., washed twice in Phage Buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 2 mM $CaCl_2$, 10 mM $MgSO_4$) to remove Tween and resuspended in 1 ml of Phage Buffer. Indicator plates were prepared by mixing 10^7 c.f.u. of fresh cultures of each of the strains with 4 ml of top agar [0.6 % w/v agar in Middlebrook 7H9 broth supplemented with 0.5 % (v/v) glycerol and 2 mM $CaCl_2$ unless otherwise indicated] and pouring the mix on top of 7H9-Gly agar plates supplemented with 2 mM $CaCl_2$, hereafter mentioned as 7H9-Gly-Ca. The ability of the mycobacteriophages to propagate on the mutant and parental strain was assessed by spotting 10 µl aliquots of serial 1/10 dilutions of each mycobacteriophage lysate – previously amplified and titrated, having average titres of 10^{10} to 10^{11} plaque forming units ml^{-1}– on top of freshly made indicator plates followed by incubation at 30 and 37 °C for 2 days.

RESULTS

*MSMEG*_1350 encodes a methyltransferase involved in epoxy-mycolic acid biosynthesis

During our studies on the effect of TAC on mycolic acid biosynthesis in *M. smegmatis* we noticed that in spite of its intrinsic resistance to this drug, a mild growth delay was

Table 1. *M. smegmatis* mc^{2}155 putative MAMT genes, their assigned functions and status of their deletion

ORF	Assigned function	Gene deletion	Reference
MSMEG_0902	Not determined	Yes, no detectable phenotype	28]
MSMEG_0913	Addition of a methyl branch to the proximal site of α- and ε-mycolates	Yes, lacked α_2- and ε_2-mycolates	31]
MSMEG_1203	Not determined	Yes, no detectable phenotype	[28]
MSMEG_1205	Not determined	Not done	-
MSMEG_1350	Unknown	Unknown	This work
MSMEG_1351	*cis*-cyclopropanation at the proximal site of α-mycolic acids (activity is significantly enhanced at temperatures lower than 37 °C)	Yes, lacked cyclopropanated mycolates.	[28]
MSMEG_3538	Not determined	Not done	-

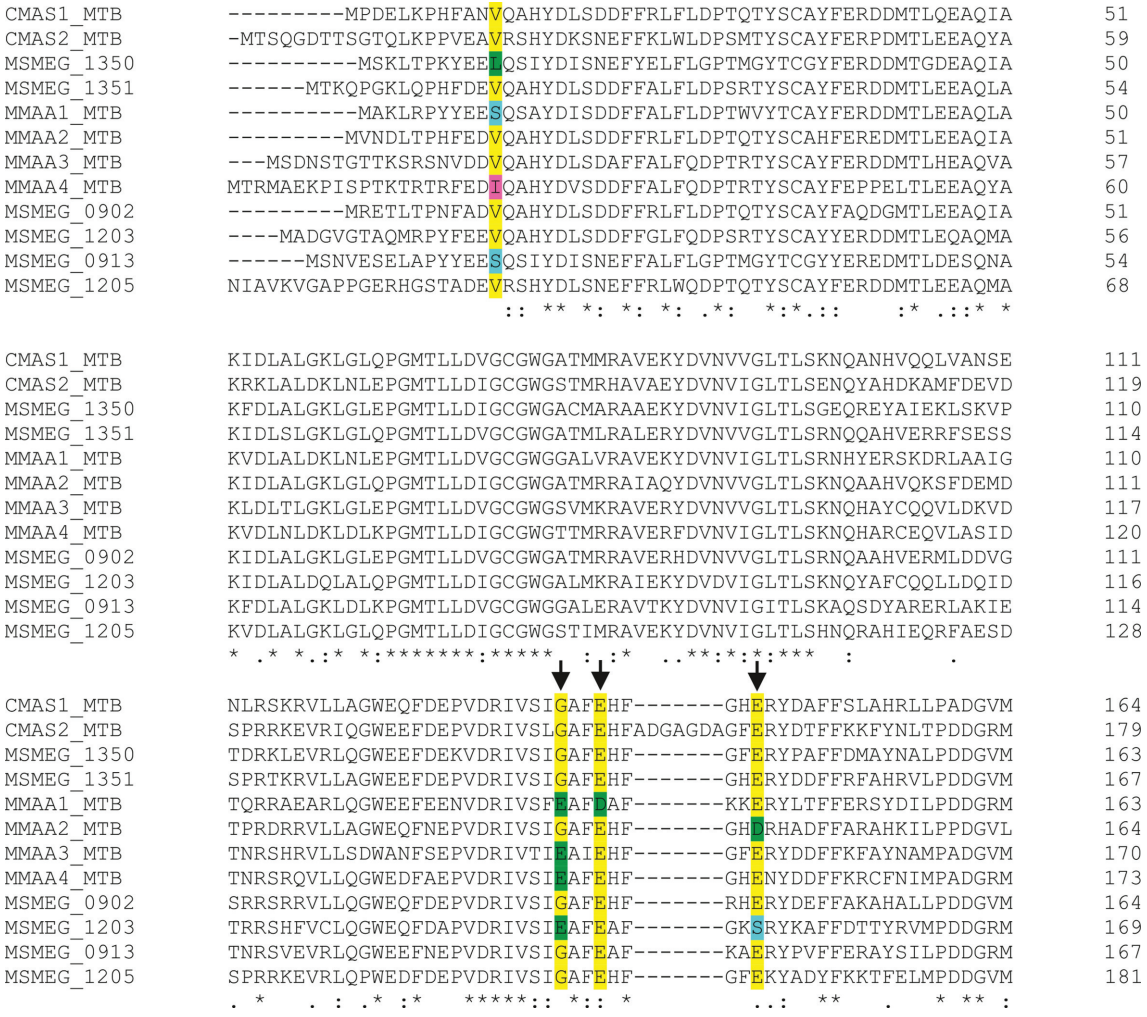

Fig. 3. Multiple amino acid sequence alignment of *M. tuberculosis* H37Rv and *M. smegmatis* mc^{2}155 methyltransferases. *M. tuberculosis* H37Rv methyltransferases included in the alignments: CMAS1_MTB (NP_217909.1); CMAS2_MTB (NP_215017.1); MMAA1_MTB (NP_215159.1); MMAA2_MTB (NP_215158.1); MMAA3_MTB (NP_215157.1); MMAA4_MTB (NP_215156.1). *M. smegmatis* mc^{2}155 methyltransferases included in the analysis: MSMEG_1350 (YP_885737.1); MSMEG_1351 (YP_885738.1); MSMEG_0902 (YP_885305.1); MSMEG_1203 (YP_885596.1); MSMEG_0913 (YP_885316.1); MSMEG_1205 (YP_885597.1). Conserved residues G140, E146 and E149 and variable residue in position 21 are denoted by arrows.

observed (Fig. 1a); thus, considering reports on the effect of TAC on MAMTs in *M. bovis* [27], we investigated the impact of the drug treatment on mycolic acids discovering that a large decrease of α_2 and ε mycolic acids was noticeable, along with the appearance of a novel spot (designated as 'z', Fig. 1b) that showed mobility comparable to a cyclopropanated mycolic acid as previously observed by Alibaud *et al.* [28]. Interestingly, the MAMT(s) behind the synthesis of α_1 is not inhibited by TAC. A survey of the *M. smegmatis* mc^{2}155 chromosome showed the presence of seven putative MAMT genes (*mma*) (Fig. 2); however, the function of only two of them (MSMEG_1351 and MSMEG_0913) was demonstrated by gene knock-out and lipid analysis of their deletion mutants (Table 1).

With the goal of identifying the role played by each MAMT in *M. smegmatis*, we first sought to identify the one(s) involved in the synthesis of epoxy-mycolates through a bioinformatics approach. Although aminoacid sequence alignment of the highly homologous MAMTs does not allow for function assignment, Defelipe *et al.* have recently performed structural and simulation studies of those enzymes in *M. tuberculosis*, proposing that specific amino acid residues were a determining factor for the outcome of each reaction and thus responsible for the nature of the final group added into the mycolic acids [29]. Analysis of the alignment of *M. tuberculosis* MAMTs and the *M. smegmatis* MAMTs (Fig. 3) showed that four of them (MSMEG_0913, MSMEG_1205, MSMEG_1351 and MSMEG_1350) can be

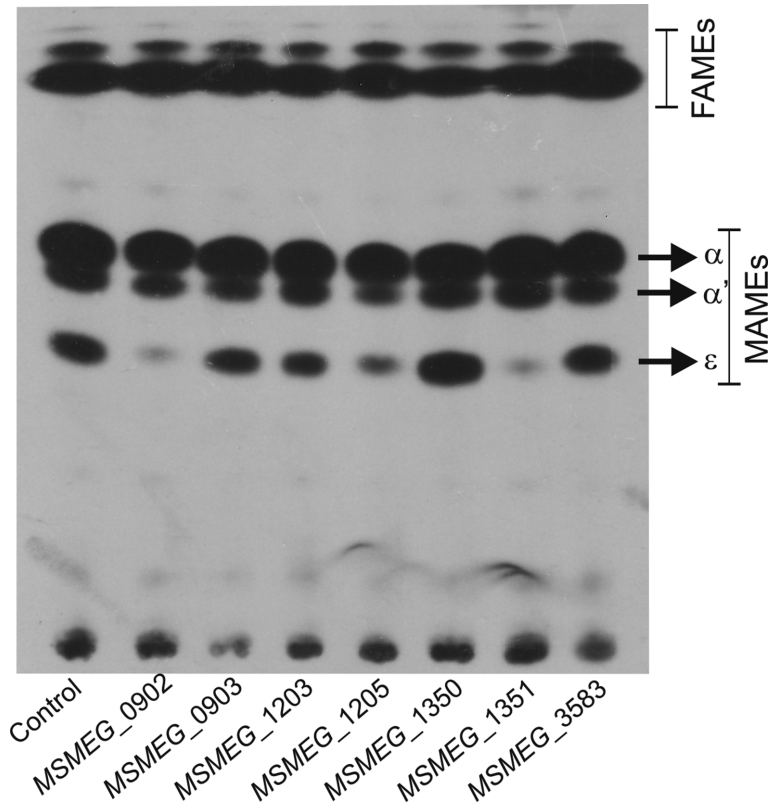

Fig. 4. Cloning and expression of putative *mma* genes in *M. smegmatis* mc^{2}155. Mid-log phase of cultures of clones expressing each *M. smegmatis* mc^{2}155 methyltransferase (MSMEG_1350, MSMEG_1351, MSMEG_0902, MSMEG_1203, MSMEG_, MSMEG_1205 and MSMEG_3583) were labelled with 1 [^{14}C] acetic acid, cells were harvested and MAMEs and FAMEs were extracted as described in Methods. The radiolabelled products were separated by TLC in silica gel 60 plates, developed three times with a mobile phase of petroleum ether-diethyl ether (17 : 3 v/v) followed by exposure to Kodak x-ray film and autoradiography at −80 °C.

grouped with the cyclopropanating *M. tuberculosis* enzymes CmaA1 and CmaA2, as they share the critical residues G140, E146 and E149 (the position number slightly differs for each enzyme) [29]. Another important residue, V21, is present in MSMEG_1205 and MSMEG_1351, matching what is found in CmaA1, CmaA2, MmaA3 and MmaA2; while MSMEG_1350 shows a Leu residue in that position and MSMEG_0913 has a S21 replacement. *M. tuberculosis* MmaA1, which introduces a methyl group adjacent to an olefinic bond, contains a V21S replacement, thus suggesting that MSMEG_0913 may perform the same task. *M. tuberculosis* MmaA4 – that leads to the introduction of a hydroxyl group – contains an V21I change, similar to the V21L change observed in MSMEG_1350. Since it is not known whether cyclopropanation occurs in a single step or in two steps, the first one being a hydroxylation – as happens with the action of MmaA4 and MmaA3 in the synthesis of methoxy-mycolates in *M. tuberculosis* [30] – both MSMEG_1205 and MSMEG_1350 could be potential candidates for that reaction.

Taking a different approach we cloned and expressed the seven *M. smegmatis* putative *mma* genes (*MSMEG*_0902, *MSMEG*_0913, *MSMEG*_1203, *MSMEG*_1205, *MSMEG*_1350, *MSMEG*_1351 and *MSMEG*_3538) in *M. smegmatis* mc^{2}155, finding that only the clone expressing *MSMEG*_1350 led to augmented synthesis of epoxy-mycolates (42 % increase over the wild-type strain) as determined by radioactivity counting of the spots normalized against the total counts present in the mycolic acids extracted in each case (Fig. 4). The other possible candidate, *MSMEG*_1205, failed to increase the content of epoxy-mycolates. Based on those observations, we deleted *MSMEG*_1350 by using a temperature-sensitive plasmid; the deletion was confirmed by PCR (Fig. S1, available with the online version of this article). *In vivo* labelling and analysis of the extracted fatty acids revealed that, as expected, epoxy-mycolates were absent in the mutant as judged by TLC (Fig. 5a). Complementation of the mutant with the integrative vector pML1342 containing the *MSMEG*_1350 gene led to restoration of the synthesis of epoxy-mycolates conclusively showing that this gene encodes a MAMT enzyme involved in that synthesis (Fig. 5a). Moreover, detailed analysis by 2D TLC showed that all three epoxy-mycolic acid species reported and structurally analysed by Laval *et al.* were missing at both 20 and 37 °C [31].

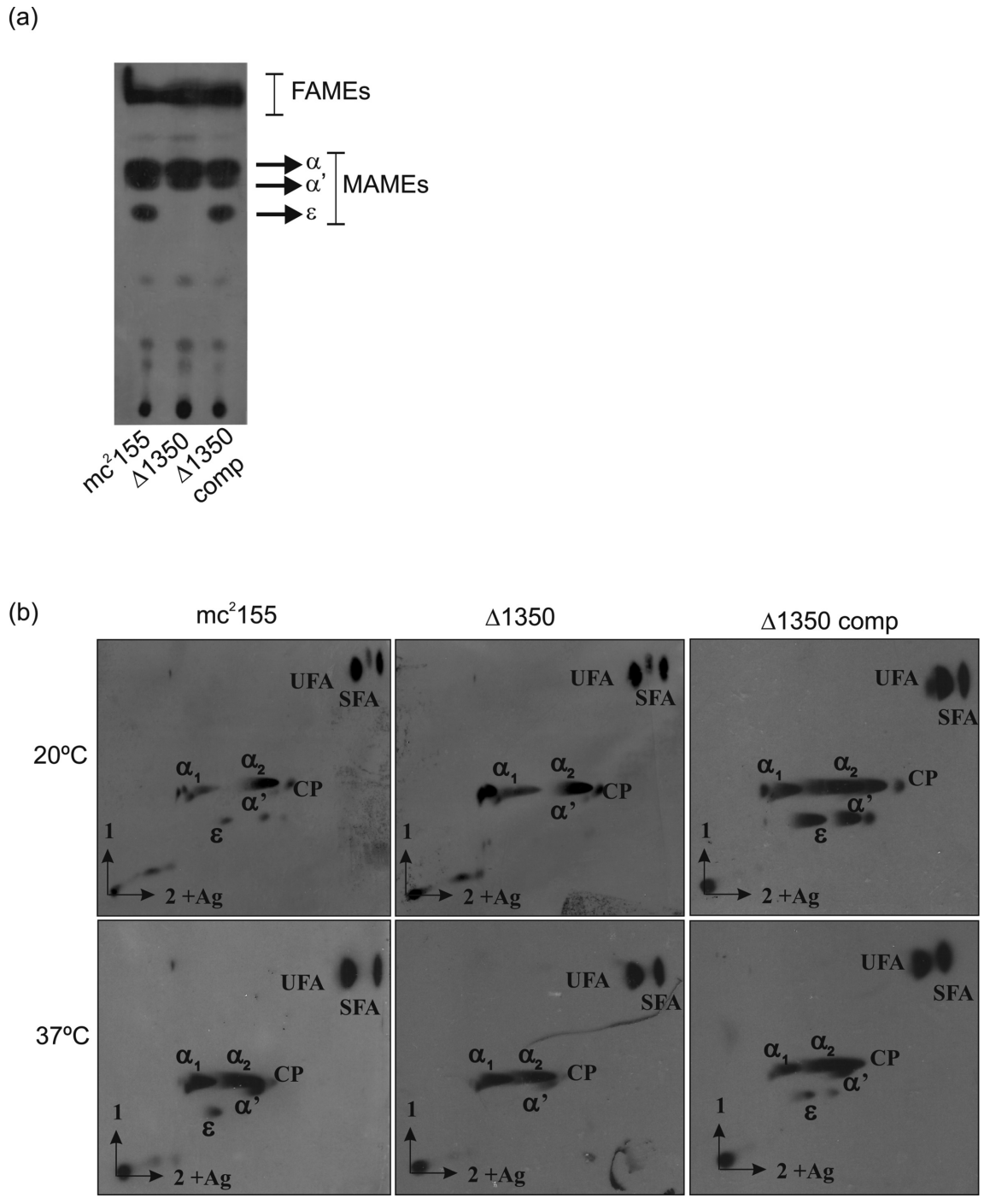

Fig. 5. Absence of epoxy-mycolic acid synthesis in the *MSMEG_*1350 deleted mutant. (a) *M. smegmatis* mc^{2}155 cultures were grown to OD_{600nm}=0.5 and radiolabelled with 1 [^{14}C] acetic acid for 3 h at 37 °C. Extracted MAMEs and FAMEs were loaded on a silica gel 60 TLC plate and developed three times with petroleum ether-diethyl ether (17 : 3 v/v) before autoradiography. (b) 1 [^{14}C] acetic acid radiolabelled cultures of parental and deleted mutant strains grown at 20 or 37 °C were pelleted and lipids were extracted as explained in Methods. 2D-TLC plates were loaded and developed with hexane-ethyl acetate (95 : 5 v/v) three times (first direction) and twice with petroleum ether-diethyl ether (17 : 3 v/v) (second direction). For both experiments, autoradiograms were obtained exposing the TLC plates to Kodak x-ray films for 24–48 h at −80 °C. CP (cyclopropanated mycolic acids), SFA (saturated fatty acids) and UFA (unsaturated fatty acids) are marked.

Importantly, under low-temperature conditions in which the synthesis of epoxy-mycolates is more evident, the

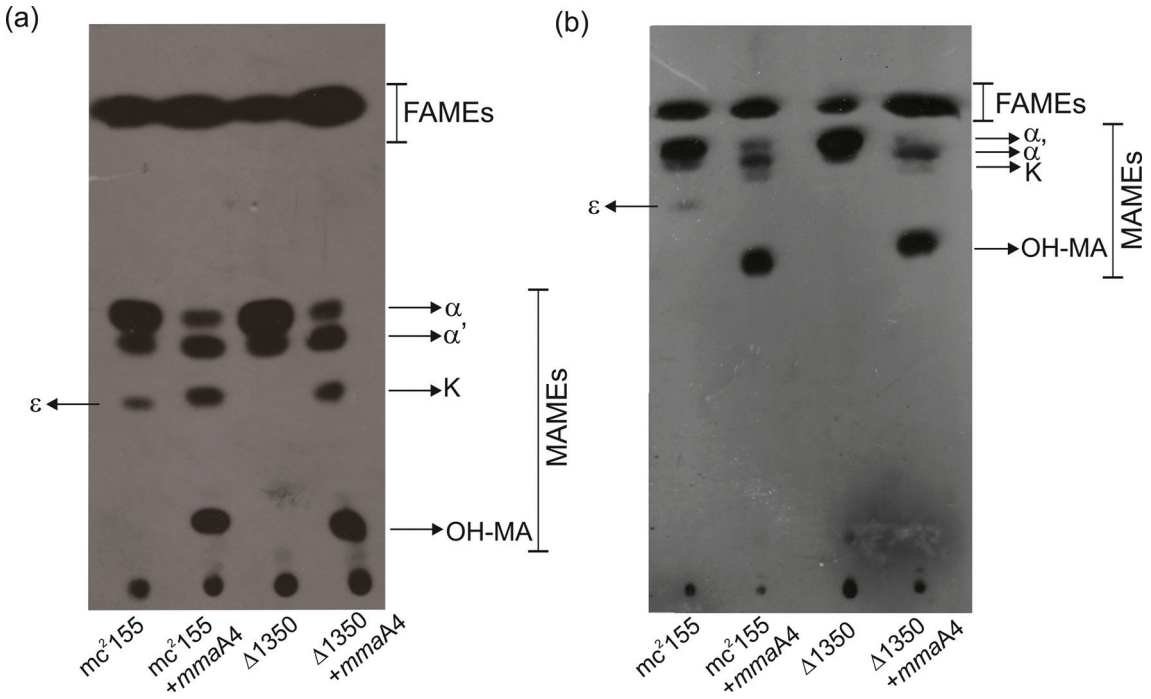

Fig. 6. Expression of *M. tuberculosis* H37Rv *mmaA4* gene in *M. smegmatis mc²155* and *M. smegmatis* Δ*MSMEG*_1350 does not restore synthesis of epoxy-mycolates. Cultures of each strain were grown to OD_{600nm}=0.5 and 1 [^{14}C] acetic acid was added. After 3 h at 37 °C, cells were harvested and lipids extracted as described in Methods. Lipid extract aliquots (50 000 cpm) were applied to silica gel TLC plates and developed by (a) three times in hexane-ethyl acetate (90 : 10, vol/vol) or (b) TLC was twice in CH_2Cl_2. FAMEs and MAMEs were designated with arrows, OH-MA, hydroxylated mycolic acids, K, keto-mycolic acids.

synthesis of cyclopropanated mycolates was not affected (Fig. 5b).

In order to gain insight on whether the synthesis of epoxy-mycolates is carried out by the sequential activity of two enzymes, the first of which would introduce a hydroxyl group, we analysed the effect of the expression of *M. tuberculosis mma*A4 in both the parental *M. smegmatis* mc²155 and the Δ*MSMEG*_1350 mutant. Separation of *in vivo* labelled fatty acids by TLC using hexane/ethyl acetate (90 : 10 v/v) (Fig. 6a) or dichloromethane (Fig. 6b) as the mobile phase showed that expression of *M. tuberculosis mma*A4 in both parental and mutant strains caused a large decrease in α-mycolates with an equivalent increase in a less mobile band with a Rf comparable to that reported in the literature for hydroxy-mycolates [8]. Simultaneously, a faint band with mobility compatible to that reported for keto-mycolates appeared below the α'-mycolates band [8]. The synthesis of epoxy-mycolates was abrogated in all conditions except for the parental strain carrying the empty pML1342 vector, which showed the normal content for those mycolates (Fig. 6a, b). Since MSMEG_1350 does not catalyse only an initial hydroxylation reaction and it is the sole enzyme accountable for carrying out the synthesis of epoxy-mycolic acids we herein propose to rename the gene as epoxy-mycolate synthase (*ems*).

Impact of the loss of epoxy-mycolates on *M. smegmatis* growth and cell-envelope features

Epoxy-mycolates are the least abundant of the three major mycolic acid families (in addition to α and α') and their role in the mycobacterial physiology has not been addressed until now. A common observation reported in several publications is that the content of epoxy-mycolates increases at low temperatures, suggesting a role in cell-envelope fluidity adaptation to temperature [32, 33]. The deletion of *ems* allowed us to test the changes that take place in the cell upon the gene loss. In the first place, we analysed the mutant ability to grow at 20, 25, 30, 37 and 42 °C in solid Middlebrook 7H9-ADS-Gly-Congo Red plates (added to improve the visualization of colony morphology changes) [16]. Interestingly, *M. smegmatis* Δ*ems* reproducibly yielded smaller, smoother and less convoluted colonies than the parental strain at either growth temperature; the changes being more noticeable at low temperatures (Fig. 7a, for simplicity only pictures obtained from cultures grown at 20, 37 and 42 °C are shown). Microscopic observation of both Ziehl–Neelsen or Gram stained aliquots of mutant and parental cultures were indistinguishable (data not shown), thus discarding gross alterations in cell size or cell-envelope organization. On the basis of those results, we next analysed the impact of the deletion of *ems* in liquid cultures using 7H9-ADS-Gly-Tr 0.25 % medium; our results demonstrate

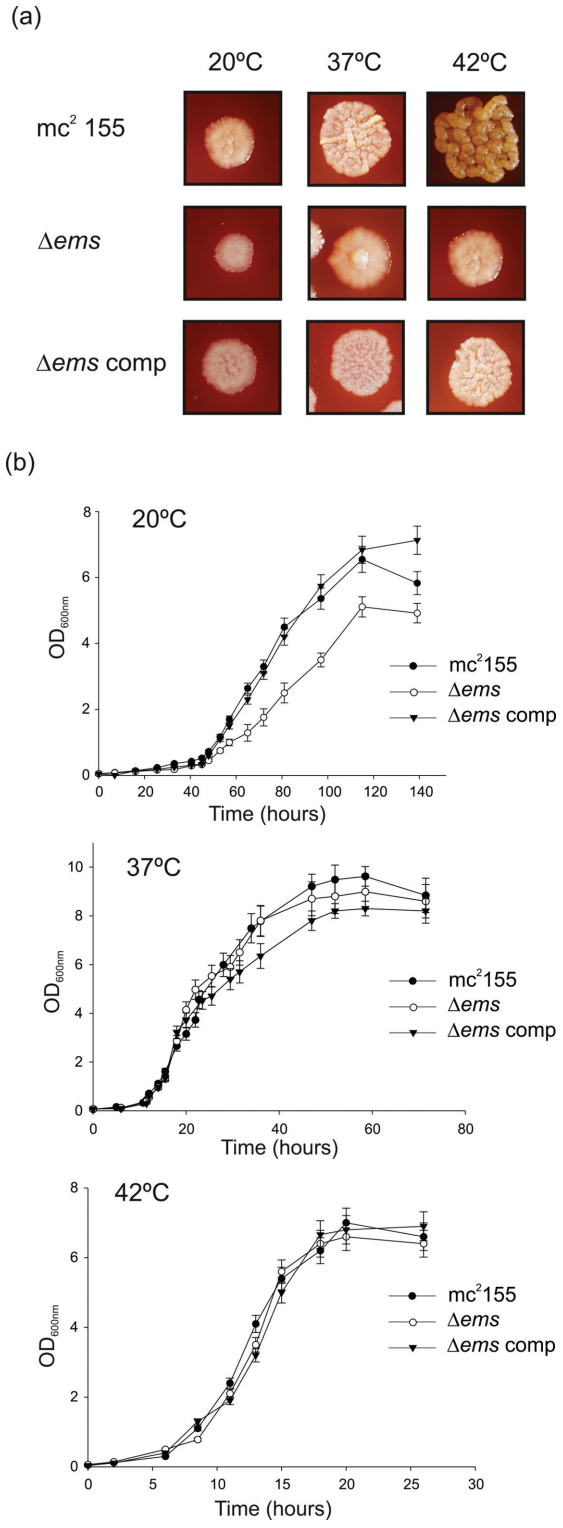

Fig. 7. Lack of epoxy-mycolates diminishes the growth rate of *M. smegmatis* at low temperature and causes colony morphology alterations. Growth of *M. smegmatis* strains mc^{2}155 and Δ*ems* on solid and liquid medium. (a) *M. smegmatis* strains mc^{2}155, Δ*ems* and Δ*ems*::pML1342*ems* (Δ*ems* comp) were grown on 7H9-ADS-Gly agar plates supplemented with Congo Red dye for 5–7 days at 20, 37 and 42 °C, colonies were observed visually and photographed with a Zeiss-Stemi 2000 lens at x2. (b) Growth curves of *M. smegmatis* strains mc^{2}155, Δ*ems* and (Δ*ems* comp) were carried out on 7H9-ADS-Gly-Tr 0.25 % medium at 20, 37 and 42 °C. (˜), *M. smegmatis* mc^{2}155 wild-type strain; (™), Δ*ems* strain, (q); complemented Δ*ems* strain.

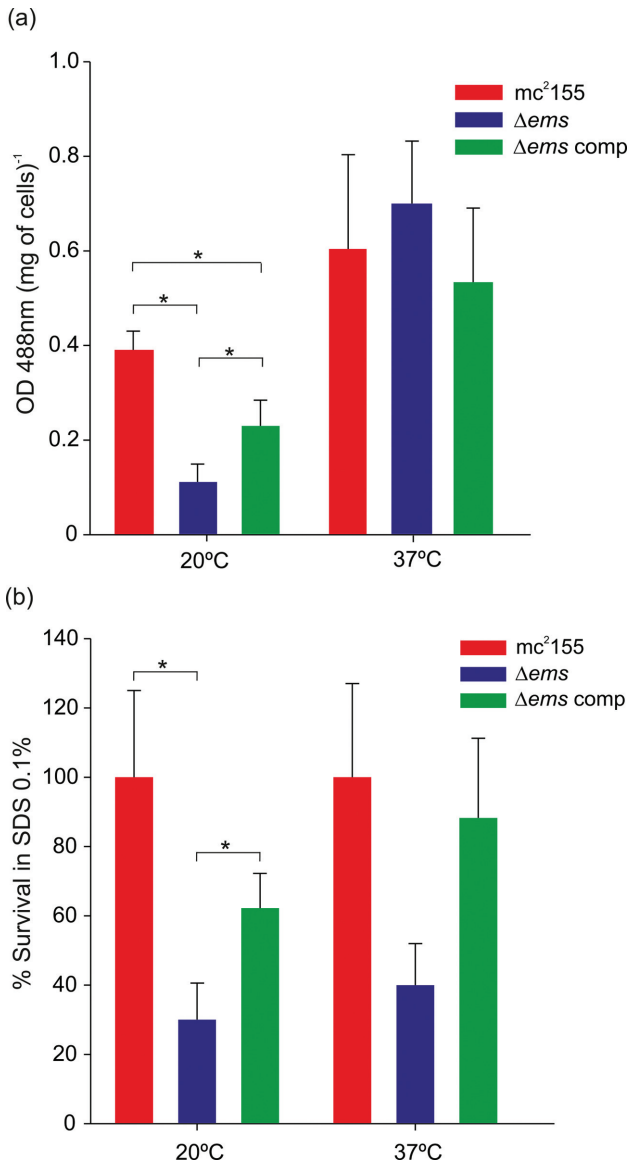

Fig. 8. Loss of epoxy-mycolates reduces Congo Red binding and survival to SDS exposure in *M. smegmatis*. (a) Cultures were grown at 20 or 37 °C in medium supplemented with Congo Red dye (100 μg ml^{-1}) for 3–5 days. Cells were collected and washed as detailed in Methods. The washed pellets were resuspended in acetone and Abs_{488} of Congo Red measured. Binding was expressed as OD_{488} pellet/dry wt. (b) *M. smegmatis* strains were grown at either 20 or 37 °C to mid-exponential phase, exposed to SDS 0.1 % (w/v) and further incubated for 3 h before plating of serial dilution and plating. c.f.u. were counted after 3–5 days incubation at the corresponding temperature. Percentages of survival after treatment were plotted considering mc^{2}155 survival as 100 %. Shown are *M. smegmatis* mc^{2}155 (red bars); Δ*ems* (blue bars) and Δ*ems* comp (green bars). The (*) indicates statistically significant differences (P<0.05).

that the loss of epoxy-mycolic acids caused a decrease in the growth rate at 20 °C in the mutant when compared to the parental strain (generation times 11 and 6 h, respectively), while the effect was negligible at higher temperatures

Table 2. Antibiotic efficiency against *M. smegmatis* mc^{2}155 (parental strain) and *M. smegmatis* Δ*ems*. MW, molecular weight; LogP, octanol/water partition coefficient, used as a measure of molecular lipophilicity. Data was extracted from https://pubchem.ncbi.nlm.nih.gov/

	MIC (μg ml^{-1}) 20 °C		MIC (μg ml^{-1}) 37 °C	
	mc^{2}155	Δ*ems*	mc^{2}155	Δ*ems*
NOV	32	16	64	32
RIF	32	8	64	32
VAN	1.5	0.5	5	5
INH	2.5	1	5	5
ERY	5	2.5	>100	>100
TAC	>100	>100	>100	>100
CV	16	16	16	32

(Fig. 7b). No differences were seen when Tw was used instead of Tr (data not shown). Complementation of the genetic defect with an integrative plasmid carrying a copy of the wild-type *ems* gene restored the growth rate to parental values at all temperatures (Fig. 7b).

Changes in the cell envelope of mycobacteria have been shown to alter aggregation of cells, biofilm formation and sliding motility [19]. A lower hydrophobicity of the cell surface would negatively affect both the sliding motility and biofilm formation due to increased interactions between the cell envelope and the hydrophilic surface of the solid medium, likewise, cell–cell contacts may be reduced, leading to lower sedimentation rates. Since loss of epoxy-mycolates seems to affect colony morphology we evaluated the impact of the loss of epoxy-mycolates in the cell-envelope properties. Our results showed that there was no significant difference in those assays at either temperature compared to the behaviour of the parental strain, suggesting that there is little modification of the hydrophobicity of the cell envelope (data not shown). However, binding of Congo Red, a hydrophobic dye that binds to lipids and lipoproteins present on the mycobacterial surface [16] was reduced 60 % in the mutant grown at 20 °C; although binding at 37 °C was comparable in both strains (Fig. 8a). This is in agreement with the fact that the content of epoxy-mycolates is higher at low temperatures, thus their loss affects the cell envelope more drastically.

M. smegmatis Δ*ems* displays an increased susceptibility to drugs and SDS

M. smegmatis mutants deficient in mycolic acid families have been reported to be more susceptible to drugs of different characteristics such as the lipophilic CV and RIF or the large hydrophilic VAN and ERY [19, 33, 34], thus we determined the MIC at 37 and 20 °C for those drugs in our mutant and the parental strains. Our results showed a slight (two to fourfold) but highly reproducible reduction in the MIC values obtained for the mutant strain compared to those of the parental one, with differences being more noticeable when the tests were performed at low

Table 3. Loss of epoxy-mycolates reduces the plating efficiency of a group of mycobacteriophages

Mycobacteriophages	Δ*ems* *	Cluster †
Che8	2	F
D29/Bxb1/21AS/L5	1	A
Bxz1	1	C
40BC/Jolie1/Hosp	1	B
19ES	1	singleton
Che9d	0,66	F
39HC	0,1	B
Jolie2	$<10^{-7}$	G
Che12/First/20ES/21AM/ 41HC/Bahia1/CRB1/Bahia2	$<10^{-9}$	A
Mine/CRB2	$<10^{-10}$	B
32HC	$<10^{-10}$	Z

*Relative plating efficiency, expressed as the ratio plating efficiency on *M. smegmatis* Δ*ems*/plating efficiency on *M. smegmatis* mc2155

†Cluster assignation was taken from [25, 41,42] and from the Actinobacteriophage database (www.phagesDB.org).

temperature. However, susceptibility to TAC remained unchanged at both temperatures while INH was more active on the mutant strain but only at 20 °C (Table 2). Taken together, our results indicate that even being a minor component of the cell envelope, the absence of epoxy-mycolates provokes changes in cell-envelope hydrophobicity or organization that leads to higher diffusion of drugs.

Chang and Cronan discovered that the loss of cyclopropanation of fatty acids in early stationary phase *E. coli* cultures led to a swift killing when abruptly shifted from neutral to acidic (pH=3) conditions [35]. Later on, *M. tuberculosis* mutants lacking cyclopropane containing mycolic acids were described, showing that their loss caused increased sensitivity to hydrogen peroxide [7]. Only a few mycobacterial species contain epoxy-mycolates including opportunistic pathogens such as *M. senegalense*, *M. farcinogenes*, *M. peregrinum* and *M. fortuitum* [36] but no other bacteria have been reported to contain epoxy-fatty acids in their cell-envelope structure. Thus, we hypothesized that mycolate epoxydation could compare to cyclopropanation in terms of conferring resistance to stress conditions. In order to determine the effect of those stress environments, we tested both the Δ*ems* mutant and the parental strain in liquid 7H9-ADS-Gly-Tw at 20 and 37 °C under acidic, osmotic, detergent or oxidative stress conditions. Our results showed that the deletion of *ems* caused a decrease in the survival of the mutant strain at both temperatures when exposed to 0.1 % SDS (ranging from 40 % at 37 °C to 75 % at 20 °C) (Fig. 8b) but not under other conditions (data not shown). Since bacterial sensitivity to detergents is usually used as a measurement of changes in the hydrophobicity of the cell envelope [19, 37] the absence of epoxy-mycolates in the cell envelope causes an alteration of its integrity and properties.

Resistance to a set of mycobacteriophages by the deletion mutant suggests modifications of the cell-wall-envelope structure originated by loss of epoxy-mycolates

As a whole, our previous results are indicative of modifications on the cell-wall structure in the *M. smegmatis* mutant devoid of epoxy-mycolates. Interactions of glycolipids (GLs) and glycopeptidolipids (GPLs) in the outer leaflet of the cell envelope depend on a normal composition of the mycolic acids exposed in its inner leaflet [38, 39]. Adsorption of bacteriophages is highly dependent of the conservation of structural components of the cell envelope defining mycobacteriophage receptors, thus we hypothesized that besides a direct change on hydrophobicity, loss of epoxy-mycolates may cause a change in the GL/GPL composition that would be easily detected by changes in the susceptibility to mycobacteriophages. Thus we tested a set of 23 mycobacteriophages that were isolated in our laboratory using *M. smegmatis* as a host as well as seven mycobacteriophages (described by others and kindly provided by G. Hatfull) [40–45]. Importantly, the micobacteriophages used belonged to different clusters, which ensured that they were genetically diverse. While all the tested mycobacteriophages were able to plate on the parental strain, yielding titres according to the original titre of each lysate, 14 of them gave faint haloes or completely failed to produce plaques on the mutant strain. Interestingly one of the phages tested behaved on the opposite way yielding larger, more turbid plaques on the mutant (Table 3 and Fig. 9).

These results suggest that the loss of epoxy-mycolates in the mutant may cause a qualitative modification in receptors for a group of mycobacteriophages leading to their inability to infect the mutant mycobacterial cell. To our knowledge this is the first report of the involvement of a single specific family of mycolic acids having a discernible role in mycobacteriophage infection, most likely during the step of phage adsorption.

DISCUSSION

An explanation for *M. smegmatis* innate resistance to TAC, involving a novel gene, has recently been published by Kremer's group [46]. However, we were intrigued by our previous findings that TAC causes a not lethal growth defect in liquid cultures of this saprophytic mycobacterial species, with inhibition of the synthesis of specific mycolate families including epoxy-mycolates indicating that specific MAMTs were inhibited (Fig. 1). *M. smegmatis* contains three families of mycolic acids, designated as α (the predominant one, accounting for ~60 %), α' (25–30 %) and epoxy-mycolates (15 % at 37 °C) [47]. Only a few mycobacterial species contain the latter mycolate, including opportunistic pathogens such as *M. senegalense*, *M. farcinogenes*, *M. peregrinum* and *M. fortuitum* [36]. Opposite to the current knowledge on the MAMTs that lead to the synthesis of cyclopropanated, keto- or methoxy-containing mycolates in *M. tuberculosis* [2, 4–6, 10, 48], there is little information on the individual

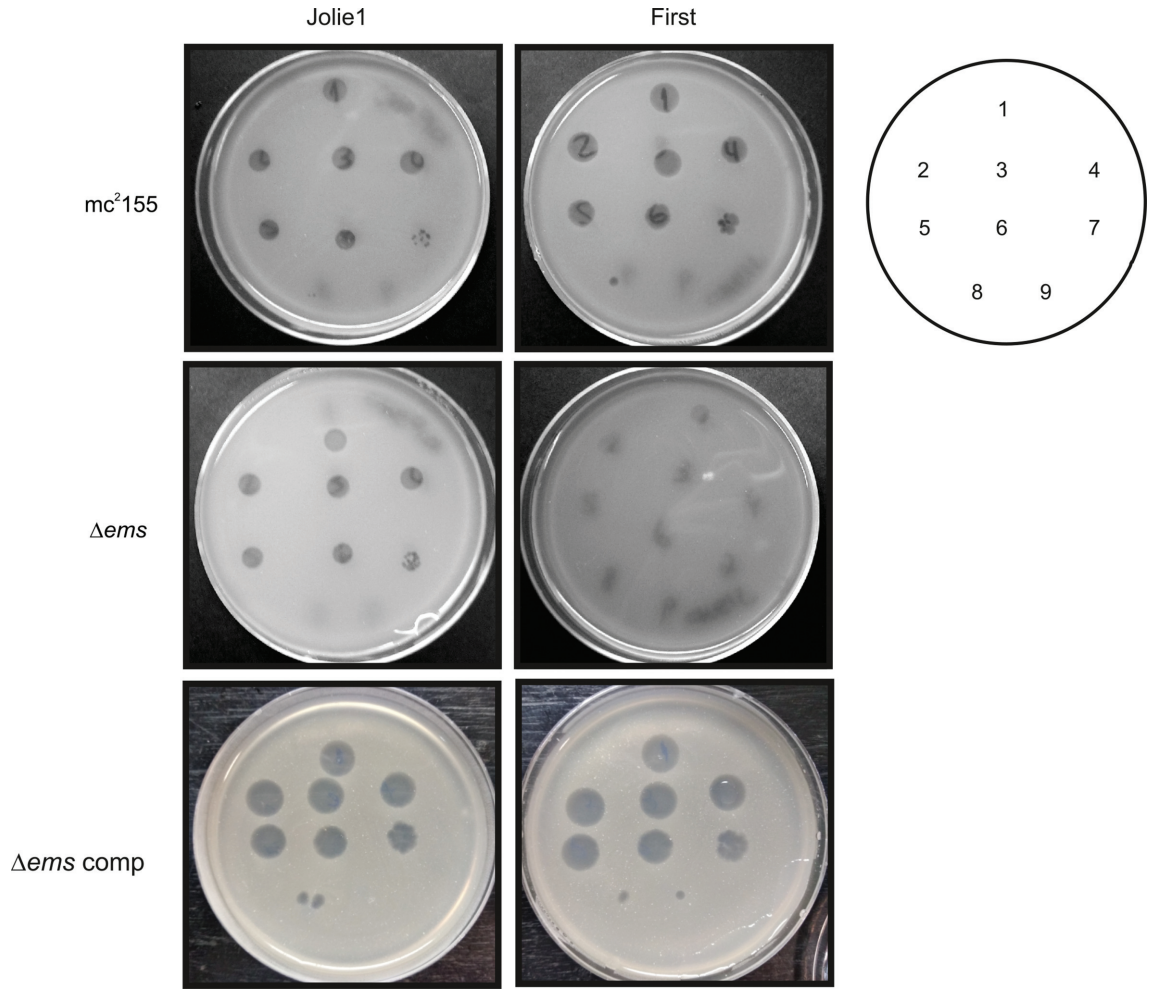

Fig. 9. Deletion of *ems* promotes resistance to a set of mycobacteriophages. Fresh cultures of parental and mutant strains (≈10^7 c.f.u.) were mixed with top agar medium and poured onto 7H9-Gly-Ca^{2+} agar plates. Aliquots (10 µl) of serial dilutions of mycobacteriophage lysates were spotted and plates incubated at 30 or 37 °C for 3 days before visual inspection. A scheme indicating the position of the serial tenfold dilutions is shown.

roles of the seven *mma* paralogues present in the *M. smegmatis* chromosome, excepting for MSMEG_0913 and MSMEG_1351 [28, 31] (Table 1, Fig. 2). The proposed reaction mechanism for MAMTs in mycobacteria involves two distinct steps; the first one, most likely identical in all MAMTs, yields a secondary carbocation on the meromycolate chain position targeted [29]. During the second step, in which the specificity of each enzyme resides, the resolution of the carbocation yields either a *cis* cyclopropane group (as seen for *cma*A1, *pca*A, *mma*A2 and *cma*A2), a *trans* double bond with a vicinal methyl group (*mma*A1) [49] or a methyl-alcohol (*mma*A4) [50] that may be converted into a methoxy group by *mma*A3 51]. Crystal structures of *M. tuberculosis cma*A1 and *cma*A2 revealed the presence of a bicarbonate/carbonate binding site, composed by His, Cys, Tyr and Gly residues [4]. It has been proposed that the bicarbonate ion is responsible for the abstraction of a proton from the added methyl group, event critical for the cyclopropane formation [29]. We found the conserved G140, E146 and E149 triad (denoted by arrows in Fig. 3) in MSMEG_1351 – a proven cyclopropanating enzyme – MSMEG_1205 and MSMEG_1350, suggesting that those enzymes may catalyse a cyclization reaction. In our hands, expression of only *MSMEG*_1350 driven by *hsp*60 in an integrative plasmid increased the contents of epoxy-mycolates while an identical construct containing *MSMEG*_1205 failed to do so (Fig. 4); moreover deletion of *MSMEG*_1350 led to the loss of all the epoxy-mycolate species in the mutant suggests that under our experimental conditions it is the only MAMT capable of catalysing the synthesis of epoxy-mycolates in *M. smegmatis* or at least the one catalysing the first step as no other intermediates were detected (Fig. 5a, b). Two mechanisms can be presented in order to explain the generation of the epoxide, one of them could be

envisioned as two enzymes acting sequentially, the first of which would introduce a hydroxyl group and the second one bringing to completion of the cyclization; the second one would require a single enzyme performing both the hydroxylation and the subsequent cyclization actions. By expressing *M. tuberculosis mma*A4 (that adds a hydroxyl group at the distal position in mycolic acids) in both parental and Δ*ems* strains, we sought to provide an extra source of hydroxylated mycolates, which may allow the suppression of the loss of MSMEG_1350 if this enzyme carried out solely a hydroxylation step. Surprisingly, expression of *mma*A4 eliminated the small synthesis of epoxy-mycolates in the parental strain, possibly reflecting the competition for the same substrate between *mma*A4 and MSMEG_1350. It is important to note that the extra amount of hydroxyl-mycolates did not restore the production of epoxy-mycolates, thus arguing against the first mechanism proposed. Taken together those results suggest that MSMEG_1350 catalyses both step(s) leading to epoxy-mycolates synthesis.

Early studies on the thermal adaptation in *M. smegmatis* pointed out that the response to low temperature caused a reduction in the chain length of α- and epoxy-mycolates and an increase in the contents of the latter with no changes in length or contents of α′-mycolates [52]; thus we hypothesized that *M. smegmatis* Δ*ems* could be impaired in growth at low temperature. Our results supported our theory as the mutant showed decreased growth rate at low temperature (20 °C) both in liquid and solid medium; causing a large increase in the doubling time (11 h for the mutant strain vs 6 h for the parental one). Moreover, colonies of the mutant strain showed morphology alterations in addition to size constraints (Fig. 7a). Interestingly, MSMEG_1351 – adjacent to MSMEG_1350 – is a cold induced α-mycolate *cis*-cyclopropanating enzyme; however its deletion did not affect growth at low temperature [42]. A plausible explanation for this different behaviour may be offered by the fact that epoxy-mycolates are more abundant at low temperature than the *cis*-cyclopropanated α-mycolates as can be observed from our results (Fig. 5) and from Alibaud's report [28]. Taken together those results suggest that epoxy-mycolates are enough to maintain *M. smegmatis* fitness at low temperature; thus a physiological role for the *cis*-cyclopropanated acids is still lacking and will most likely require the deletion of both MSMEG_1350 and MSMEG_1351 to be deciphered.

Changes in the sensitivity to SDS have often been used as an indication of an alteration of the hydrophobicity of bacterial envelopes [37]. Including those of mycobacteria [19, 53]. This was proven to be correct as the *M. smegmatis* Δ*ems* mutant was slightly more sensitive to SDS, especially at low temperature. It also displayed a low but consistent increase in susceptibility to both large hydrophilic drugs (ERY and VAN) and lipophilic drugs (RIF, NOV) and reduction of Congo Red binding. However, intriguingly, entry of a small drug such as TAC, which is slightly lipophilic (logP=1.22), is not affected in the mutant strain while INH, a more hydrophilic molecule (logP=−0.89) is more active on the mutant strain at low temperature (Table 2). As a whole, our results show that lack of epoxy-mycolates cause directly or indirectly mild but highly reproducible changes in cell-envelope hydrophobicity although other direct or indirect effects are still to be deciphered.

Surprisingly, our observation that lack of epoxy-mycolates prevented or largely decreased the efficiency of plating of several mycobacteriophages is yet another proof that although a minor component, epoxy-mycolates play a definite role in *M. smegmatis* cell-envelope structure and physiology. The chemical identity of mycobacteriophage receptors is poorly known, with only a handful of reports showing that resistance to specific mycobacteriophages is linked to changes in GPL species [54, 55]. A very elegant study recently published identified HadD as a novel component of the FASII system in *M. smegmatis*. Its deletion caused the loss of α- and epoxy-mycolates (which accounts for a large fraction of the normal cell-wall-linked mycolic acid content) as well as of polar GPLs, which translated into large increases in drug and detergent susceptibility and reduction in cell–cell aggregation among other traits assayed [39]. In the case of Δ*em*s we may assume that the loss of the relatively small fraction of the total mycolates represented by epoxy-mycolates would lead to a deficiency in the proper localization of only a small fraction of the total polar GPLs. In spite of that, the large reduction in plating efficiency shown for several mycobacteriophages strongly suggests that α-mycolates cannot replace for epoxy-mycolates in the correct localization of the otherwise loose GPLs, therefore causing the destruction of mycobacteriophage receptors from the cell surface. In this context – although speculative – it is possible that specific GPLs could interact with each mycolic acid family creating the different mycobacteriophage receptors, a hypothesis that deserves future studies.

In conclusion, our findings help to assign a function to a member of the *M. smegmatis* MAMT group and bring back to the spotlight the importance of those enzymes as a means to increase the susceptibility of *M. smegmatis* to currently available drugs.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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