Molecular Microbiology (2009) 71(5), 1263–1277 doi:10.1111/j.1365-2958.2009.06604.x
First published online 4 February 2009

Mycolic acid methyltransferase, MmaA4, is necessary for thiacetazone susceptibility in *Mycobacterium tuberculosis*

Anuradha Alahari,[1†] Laeticia Alibaud,[1] Xavier Trivelli,[2] Radhika Gupta,[3] Gyanu Lamichhane,[3] Robert C. Reynolds,[4] William R. Bishai,[3] Yann Guerardel[2] and Laurent Kremer[1,5]*

[1]*Laboratoire de Dynamique des Interactions Membranaires Normales et Pathologiques, Université de Montpellier II et I, CNRS; UMR 5235, case 107, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.*
[2]*Unité de Glycobiologie Structurale et Fonctionnelle, CNRS UMR 8576, IFR 147, Université des Sciences et Technologies de Lille, 59650 Villeneuve d'Ascq, Cédex, France.*
[3]*Center for Tuberculosis Research, Division of Infectious Diseases, Johns Hopkins University School of Medicine, Baltimore, MD 21231-1001, USA.*
[4]*Drug Discovery Division, Southern Research Institute, PO Box 55305, Birmingham, AL 35225-5305, USA.*
[5]*INSERM, DIMNP, Place Eugène Bataillon, 34095 Montpellier Cedex 05, France.*

Summary

Susceptibility of *Mycobacterium tuberculosis* to the second-line antitubercular drug thiacetazone (TAC) requires activation by the monoxygenase, EthA. Here, we report isolation of spontaneous mutants in *Mycobacterium bovis* BCG that are highly resistant to TAC, but carry a functional EthA. Unexpectedly, a majority of the TAC-resistant mutants lacked keto-mycolic acids, which are long-chain fatty acids associated with the cell wall and which contribute significantly to the physiopathology of tuberculosis. Predictably, causative mutations in the above mutants were in the gene encoding methyltransferase MmaA4, which is required for synthesis of keto- and methoxy-mycolic acids. Drug-resistant phenotype of the BCG mutants was reproduced in a *mmaA4*, but not in a *mmaA3* null mutant of *M. tuberculosis* CDC1551. Susceptibility to TAC could be restored by complementation with a functional *mmaA4* gene. Interestingly, overexpression of MmaA4 in *M. bovis* BCG made it more susceptible to TAC. We provide novel mechanistic insights into antitubercular drug activation by co-ordinated actions of EthA and MmaA4. This study is the first demonstration of the participation of an enzyme linked to the synthesis of oxygenated mycolates in a drug activation process in *M. tuberculosis*, and highlights the interplay between mycolic acid synthesis, drug activation and mycobacterial virulence.

Accepted 9 January, 2009. *For correspondence. E-mail laurent.kremer@univ-montp2.fr; Tel. (+33) 4 67 14 33 81; Fax (+33) 4 67 14 42 86. †Present address: Equipe SP2, INSERM U919, GIP Cyceron, Campus Jules Horowitz, BP 5229, Caen, France.

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd

Introduction

Mycobacteria are distinguished by the presence of a highly hydrophobic cell envelope which is thought to be largely responsible for the success of species such as *Mycobacterium tuberculosis* (*M. tb*) as intracellular, human pathogens, capable of long-term survival in a dormant form (Brennan and Nikaido, 1995; Daffe and Draper, 1998). The lipidic cell envelope of mycobacterial strains represents up to 60% of the cell's dry weight (Brennan and Nikaido, 1995). It includes the extremely long-chain fatty acids called mycolic acids, which are covalently linked to a lower layer of arabinogalactan (Kremer *et al*., 2000; Takayama *et al*., 2005). *M. tb* carries three types of mycolic acids: di-cyclopropanated α-mycolic acids and oxygenated, methoxy- and keto-mycolic acids (Fig. 1). The vaccine strain, *Mycobacterium bovis* BCG strain Pasteur (hereafter referred to as BCG), has a similar mycolic acid profile, but lacks methoxy mycolates. Synthesis of cell wall lipids, particularly mycolic acids, is essential for survival of mycobacteria *in vivo*. Several of the successful antimycobacterial drugs, including isoniazid (INH) or ethionamide (ETH), inhibit enzymes required for mycolic acid synthesis (Banerjee *et al*., 1994; Vilcheze *et al*., 2006; Wang *et al*., 2007).

Thiacetazone (TAC) is an inexpensive, second-line antitubercular and bacteriostatic drug that has been widely used in combination with INH throughout the developing world to treat patients infected with multi-drug-resistant *M. tb* strains (Davidson and Le, 1992; Nunn *et al*., 1993). Chemical analogues of TAC, SRI-224 and SRI-286, have been synthesized and tested against *Mycobacterium avium* and found to be more effective than

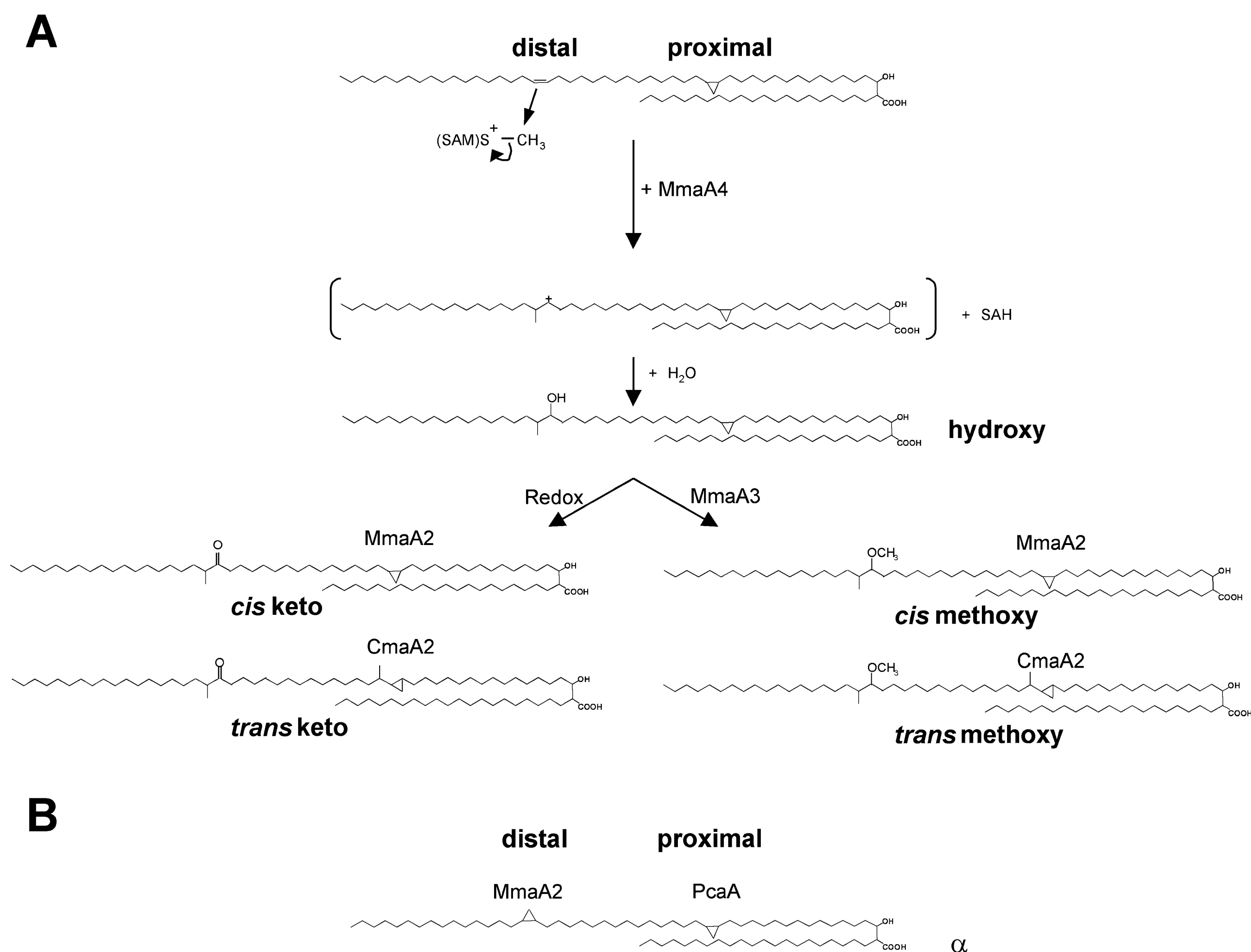

Fig. 1. Schematic representation of mycolic acids and synthesis of the oxygenated mycolic acids in *M. tuberculosis*.
A. Precursor of keto-mycolic acid carrying a proximal cyclopropane and unsaturation at the distal position is shown. MmaA4 uses the methyl group from *S*-adenosyl to generate *S*-adenosylhomocysteine (SAH) as a by-product and an intermediate, which is eventually converted to hydroxy mycolic acid. Hydroxy mycolic acid can be transformed into either keto-mycolic acid by a redox reaction or into methoxy-mycolic acid by the action of MmaA3. Depending on the orientation of the proximal cyclopropane, *cis*- or *trans*-isomers are produced. Various *S*-adenosyl-methionine (SAM)-dependent methyltransferases of the CMAS family that introduce cyclopropane functions at the proximal and distal positions in the meromycolic acid are indicated.
B. Structure of α-mycolic acid and the required CMAS enzymes. Except for MmaA4, all the other CMASs are inhibited by TAC (Alahari *et al.*, 2007). While the tranformations are shown for mycolic acids, it is likely that they occur at some stage in the synthesis of meromycolates.

TAC *in vitro* and in mice (Bermudez *et al.*, 2003). TAC has recently been chosen as a target pharmacophore for the preparation of chemical analogues, which have been evaluated for their potential in inhibiting *M. tb* H37Rv growth (Alahari *et al.*, 2007). Several of these exhibited potent antitubercular activity and, among them, a few compounds were found to be more active than the parent molecule, suggesting that newer, more effective analogues could be developed for clinical use (Alahari *et al.*, 2007).

Recently, it was shown that TAC inhibits a step in the mycolic acid biosynthetic pathway. Treatment of BCG with TAC results in diminished content of all types of mycolic acids due to inhibition of the cyclopropanating mycolic acid synthases (CMASs), which are required for modifications of the meromycolic acid precursor (Alahari *et al.*, 2007). As illustrated in Fig. 1, the CMAS family of enzymes includes CmaA2, MmaA2 and PcaA, which convert the proximal or distal double bonds of the meromycolic acids into cyclopropane rings. MmaA4 (also known as Hma) is required to produce the hydroxymycolate precursor of the oxygenated mycolic acids, while MmaA3 is responsible for *O*-methylation of hydroxymycolate precursor to form methoxy-mycolates (Yuan *et al.*, 1998; Dubnau *et al.*, 2000; Kremer *et al.*, 2000; Takayama *et al.*, 2005). The above CMASs are closely related *S*-adenosyl-methionine (SAM)-dependent methyltransferases. All of these are inhibited by TAC even at extremely low doses, with the notable exception of MmaA4. Thus, an uninhibited activity of MmaA4 in the

© 2009 The Authors

presence of TAC ensures a supply of uncyclopropanated mycolates, which replace the normal mycolates in the cell wall (Alahari *et al*., 2007).

Effectiveness of antitubercular drugs such as INH (Zhang *et al*., 1992), PA-824 (Stover *et al*., 2000; Manjunatha *et al*., 2006), pyrazinamide (Scorpio and Zhang, 1996) or ETH (Baulard *et al*., 2000; DeBarber *et al*., 2000) is governed by the presence of an activator enzyme that converts the inactive pro-drug to their active and lethal form(s). Clinically isolated drug-resistant *M. tb* strains have been found to harbour a mutation in the gene encoding the activator. Because the monooxygenase EthA is a common activator of thiocarbamide-containing drugs, such as ETH, TAC and isoxyl (ISO) (Dover *et al*., 2007), mutations in the *ethA* gene lead to high-level multi-drug resistance (DeBarber *et al*., 2000).

In this study we describe isolation and characterization of spontaneous mutants of mycobacteria resistant to TAC. Care was taken to avoid recovering mutants in *ethA* by using as parent, a strain carrying this gene on a multi-copy plasmid. Nine stably drug-resistant independent mutants were isolated. Among the mutants that were tested, all had a wild-type level of susceptibility to other drugs, such as INH or rifampicin. Five of the resistant mutants were found to lack keto-mycolic acids in the cell wall. Hence, these five were suspected, and eventually confirmed, to be mutants in *mmaA4*. Transposon-insertion mutants of *M. tb* CDC1551 in *mmaA4* and *mmaA3* were tested for their susceptibility to TAC. As in the case of BCG, a *M. tb_mmaA4* mutant was highly resistant to TAC. Complementation with a functional *mmaA4* gene reversed the drug susceptibility phenotype in these strains. Further, overexpression of *mmaA4* in BCG resulted in increased susceptibility to TAC. Taken together our data suggest that in addition to EthA, MmaA4 is required for activation of TAC.

Results

Isolation of spontaneous mutants resistant to TAC and SRI-224

In continuation of our studies related to the antitubercular activity of TAC, a screen for recovering spontaneous mutants resistant to this drug was designed. It is known that mutations in *ethA* can confer resistance to TAC (DeBarber *et al*., 2000). In order to avoid uncovering *ethA* mutants, we employed a BCG strain overexpressing a plasmid-encoded functional *ethA* gene, as a parental strain. In addition, another parental strain of BCG carrying the empty plasmid was also used. Cultures of both strains were plated on media supplemented with either TAC or SRI-224 at concentrations that were at least 4–10 times higher than the minimum inhibitory concentration (MIC) for parental BCG cultures. Colonies recovered from these plates were presumably spontaneous mutants highly resistant to TAC (Table 1). Altogether, from the two parental strains, 12 mutants were recovered and labelled R1 through R12. These included some that overexpress *ethA* (R1 through R5) and others that do not (R6 through R12) and exhibited varying degrees of resistance to the drug (Table 2). Of these, R1 subsequently reverted to wild-type phenotype on repeated subculture in medium lacking TAC and was omitted from the study. A few were found to be siblings, so that eventually there were nine independent and stable TAC-resistant mutants in the collection. Table 2 describes the mutant strains generated in this study, indicating concentration of the drug on which these were

Table 1. Mycobacterial strains and plasmids used in this study.

Strain/plasmid	Description	Marker
Strain		
M. bovis BCG	Strain Pasteur 1173P2	–
R(#)	Spontaneous mutants of BCG, resistant to TAC	Km
R(#)/361_mmaA4	Above mutants complemented with *M. tb_mmaA4* gene	Km, Hyg
M. tuberculosis	Strain CDC1551	–
JHU0643c-459	CDC1551 *mmaA3::Himar1*	Km
JHU0642c-583	CDC1551 *mmaA4::Himar1*	Km
JHU0642c-583/361_mmaA4	JHU0642c-583 complemented with pMV361_*M. tb_mmaA4*	Km, Hyg
Plasmid		
pMV261	Multi-copy shuttle plasmid for *Escherichia coli* and mycobacteria	Km
pMV361	Plasmid allows single-copy integration of cloned gene at the *attB* site on the genome	Hyg
261_mmaA4	*M. tb_mmaA4* gene cloned in pMV261	Km
361_mmaA4	*M. tb_mmaA4* gene cloned in pMV361	Hyg
261_mmaA3	*M. tb_mmaA3* gene cloned in pMV261	Km
361_mmaA3	*M. tb_mmaA3* gene cloned in pMV361	Hyg
261_ethA	*M. tb_ethA* gene cloned in pMV261	Km

All *M. bovis* BCG mutant strains described in this study are kanamycin resistant due to presence of either pMV261 or pMV261_*ethA*.
Km, kanamycin resistance; Hyg, hygromycin resistance.

© 2009 The Authors

Table 2. Characteristics of the parental and spontaneous mutant strains of *M. bovis* BCG.

M. bovis BCG strains	Selection on drug (μg ml^{-1})	MIC TAC (μg ml^{-1})	MIC SRI-224 (μg ml^{-1})	*mmaA4*, nt	MmaA4, aa	keto-mycolates
Parental strain (pMV261)	–	0.5	0.25	wt	wt	Yes
R6	TAC, 2.5	10	10	Substitution g169a	A57T	No
R7	TAC, 2.5	10–25	10–25	Deletion t275	STOP	No
R8	TAC, 2.5	10	10	wt	wt	Yes
R10	TAC, 2.5	10–25	10–25	Substitution g131t	C44F	No
R11	TAC, 1.5	10–25	10–25	wt	wt	Yes
R12	TAC, 1.5	10–25	10–25	Deletion a724	STOP	No
Parental strain (pMV261_*ethA*)	–	0.5	0.25	wt	wt	Yes
R2	SRI-224, 1.5	25	25	Substitution g254a	G85D	No
R4	SRI-224, 1.0	1–2.5	1–2.5	wt	wt	Yes
R5	SRI-224, 1.0	1–2.5	1–2.5	wt	wt	Yes

originally selected. For all mutant strains, MIC values of TAC or its related analogue SRI-224 (Bermudez *et al.*, 2003; Alahari *et al.*, 2007) were comparable. It can be seen that mutant strains overexpressing *ethA* or not could be equally highly resistant to TAC.

Altered mycolic acid profile in TAC-resistant mutants

Inhibition of cyclopropanation of mycolic acids is one of the effects of treatment with TAC or its analogue SRI-224 (Alahari *et al.*, 2007). Hence, we examined the mycolic acid profile of all the mutant strains (Fig. 2). Cells in exponential phase of growth were either treated or not with high doses of TAC or SRI-224 (10 μg ml^{-1}) and labelled with ^{14}C-acetate. Fatty acid methyl esters (FAMEs) and mycolic acid methyl esters (MAMEs) were extracted, resolved by thin-layer chromatography (TLC) and visualized by autoradiography as described earlier (Dover *et al.*, 2007). Resolution of extracts by conventional TLC revealed that five of the nine mutants (R2, R6, R7, R10 and R12) presented an unusual mycolic acid profile characterized by a complete loss of keto-mycolates and synthesis of only α-mycolates (Fig. 2A). This peculiar mycolate profile is suggestive of a loss of MmaA4 activity, which is required for the synthesis of oxygenated mycolic acids (Dubnau *et al.*, 2000). Strains R4 and R5 produced both α- and keto-mycolates, but the overall mycolic acid content was reproducibly lower than in the parental strain (Fig. 2A), whereas in strain R11, the ratio of α- : keto-mycolates appeared to be reversed compared with the parental *M. bovis* BCG strain. R8 is a notable exception in this collection because it is highly resistant to TAC while exhibiting a mycolic acid profile comparable to its parental strain (Fig. 2A and Table 2).

More subtle differences were apparent when lipid extracts were resolved on two-dimensional TLC (2D-TLC) plates impregnated with silver nitrate. This method of argentation TLC retards the migration of unsaturated FAMEs and MAMEs (Kremer *et al.*, 2002). TLC analysis of untreated cultures of R2 and R7 mutants revealed the presence of an additional spot (termed y1) running along with di-cyclopropanated α-mycolates, with y1 being the major form, compared with that in cultures of untreated parental strain (Fig. 2B). Following treatment with high doses of SRI-224, di-cyclopropanated α-mycolate synthesis was completely abrogated, with a concomitant accumulation of y2 (Fig. 2B). The y1 and y2 forms presumably correspond to mono- and di-unsaturated α-mycolates precursors. As reported earlier for parental BCG strain, there was a significant reduction in synthesis of α-mycolates and to a lesser extent in that of keto-mycolates even at low drug concentrations (Fig. 2B) (Alahari *et al.*, 2007).

Importantly, in these strains, a characteristic diminution of mycolic acids due to inhibitory effect of TAC on CMASs was observed. This was true for mutants that produced keto-mycolates as well as for those that did not. The expected accumulation of uncyclopropanated forms of mycolates was also seen (labelled x or y2 in Fig. 2B). This indicates that mutants were permeable to high concentrations of SRI-224 (or TAC) and that the drug resistance was not due to changes in cell wall permeability. Second, the data indicate that EthA is functional in these mutants since the EthA-activated form of TAC is required to inhibit the CMAS enzymes (Dover *et al.*, 2007).

Structural analyses of mycolic acids in TAC-resistant mutants

Structural modifications of mycolates observed in SRI-224 or TAC-resistant mutant strains were further confirmed by mass spectrometry (MS) and nuclear magnetic resonance (NMR) analyses. Mycolates were extracted from cultures of parental BCG strain and R2 mutant that lacks keto-mycolates. The different types of MAMEs from the extract were isolated by separation on preparative TLC and subjected to MS analysis. Whereas the parental strain was characterized by a single family of α-mycolates with an even number of carbons differing from one another by 28 mu (C_{76} to C_{82}), R2 showed a set of signals

© 2009 The Authors

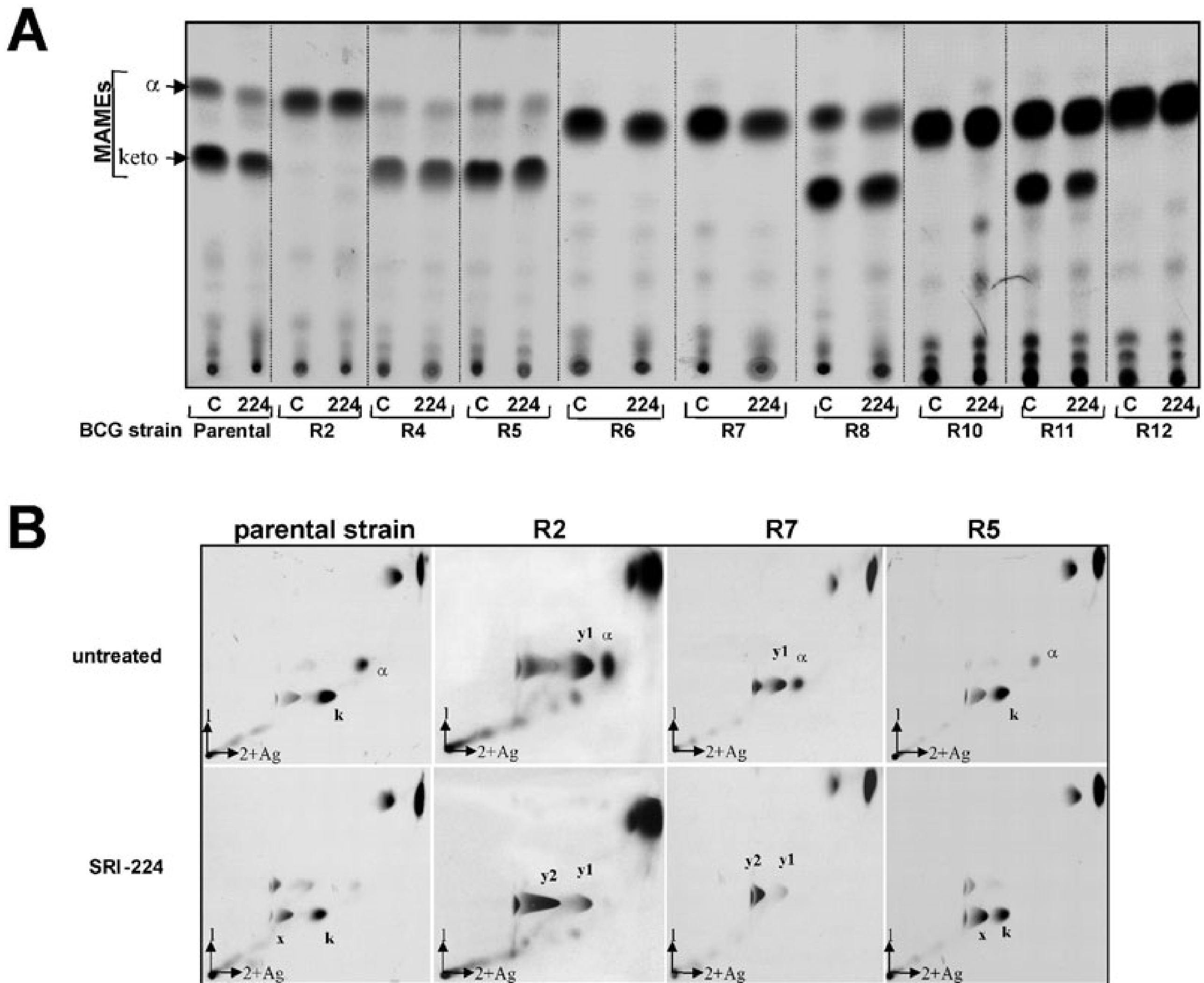

Fig. 2. Mycolic acid profile from various TAC-resistant mutants of *M. bovis* BCG. ^{14}C-labeled cultures were either treated with 10 μg ml^{-1} SRI-224 (224) or not (C) and mycolates extracted from these were resolved by single dimension TLC (A), or by two-dimensional TLC on silver nitrate-impregnated plates (B). α, α-mycolates; keto, keto-mycolates; y1 and y2 correspond to mono- or di-unsaturated precursors of α-mycolates respectively;
x, uncyclopropanated precursor of keto-mycolates.

differing by 14 mu, establishing the presence of two families with even and odd numbers of carbons (C_{76} to C_{82} and C_{77} to C_{83}) (Fig. 3A, top). Thus, as observed by 2D-TLC, R2 α-mycolates are a mixture of di-cyclopropanated and mono-cyclopropanated molecules (labelled α and y1 in Fig. 2B). The latter accumulate presumably due to the absence of keto-mycolates. Following treatment with SRI-224, R2 showed a mixture of α-type mycolates with even and odd numbers of carbons, but characterized by a shift towards lower molecular masses due to an average loss of two carbons (C_{73} to C_{81}) (Fig. 3A, bottom). Again, these data support disappearance of α-mycolate with almost exclusive accumulation of y2-mycolates, as observed by 2D-TLC (Fig. 2B). Unexpectedly, MALDI-MS spectrum of SRI-224-treated R2 exhibited a third yet unidentified family of mycolates that differ from other families by a mass increment of 2 mu. Such a mass difference may arise from hydrogenation of an unsaturated bond. However, the precise structures of these potentially modified mycolic acids need to be further characterized.

Mass spectrometry analysis demonstrated changes in the pool of unsaturated and cyclopropyl functions in the α series of mycolic acids in parental R2 strain after exposure to SRI-224. This was further substantiated by NMR spectroscopy that enabled quantification of relative intensities of *cis*-cyclopropyl and ethylenic proton resonance signals, according to published parameters (Alahari *et al.*, 2007) (Fig. 3B). It was seen that relative amount of unsaturated molecules (indicated by the ethylenic proton resonance signal) increased due to treatment with the drug.

© 2009 The Authors

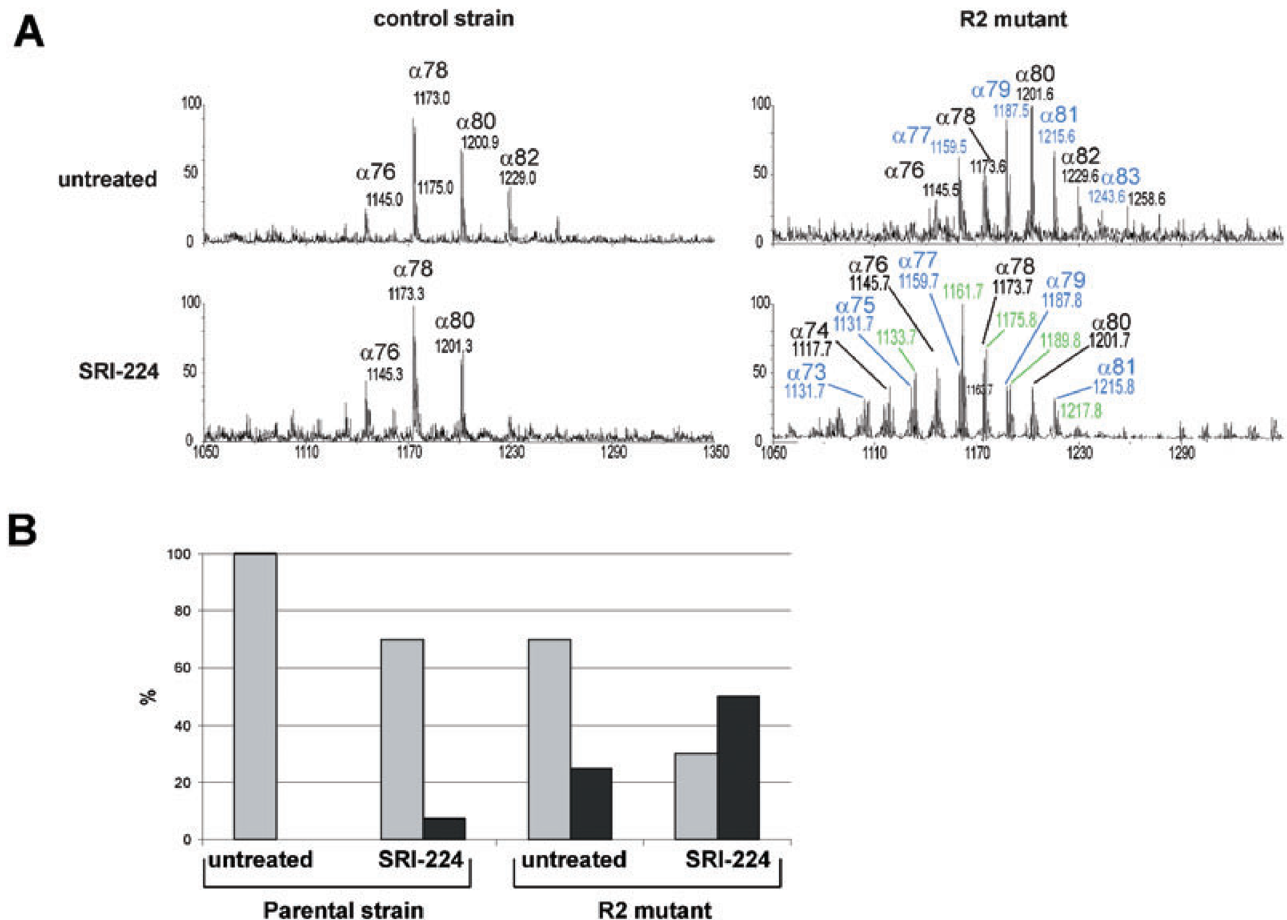

Fig. 3. Structural analysis of purified mycolates from parental strain or R2 mutant.
A. MALDI-MS profiles of MAMEs from the indicated strain, either treated (bottom) or not (top) with 10 μg ml^{-1} SRI-224. In black, α-mycolates with even number of carbons; in blue, α-mycolates with odd number of carbons, presumably from y1; in green, a yet unidentified α class of mycolates found in R2 treated with SRI-224.
B. Relative quantification by integration of NMR signals from protons from *cis*-cyclopropyl (grey bars) and ethylenic groups (black bars, indicative of unsaturation) in MAMEs purified from the parental strain, BCG/pMV261-*ethA* or R2 mutant either untreated or treated with 10 μg ml^{-1} SRI-224, as indicated.

Also, the R2 mutant carries a higher percentage of unsaturated molecules, even without drug treatment, which is consistent with observations made in a *mmaA4* (*hma*) mutant of *M. tb* (Laval *et al*., 2001; Dinadayala *et al*., 2003). This is probably indicative of accumulation of precursor molecules as a result of a lack of synthesis of oxygenated mycolates.

Resistance to TAC is associated with mutations in mmaA4

Absence of keto-mycolates in TAC-resistant strains prompted us to investigate whether this phenotype was linked to lesions in the *mmaA4* gene. Sequence of *mmaA4* was examined in all mutant strains by PCR amplification of this genomic region. Nucleotide sequence analysis revealed that all those mutants that lacked keto-mycolates had an altered *mmaA4* sequence (Table 2), while others carried a wild-type *mmaA4* gene. Strains R2 through R5 were derived from parental strain BCG/pMV261_*ethA*, while R6 through R12 were derived from BCG/pMV261. Strains R2, R6 and R10 possess a missense mutation (G85D, A57T and C44F respectively) in the *mmaA4* gene (Fig. 4). Interestingly, all three mutations were located in the region encoding the N-terminus of the protein. The three mutated residues (Cys44, Ala57 and Gly85) are conserved in the eight members comprising the SAM-dependent methyltransferase family in *M. tb* (Boissier *et al*., 2006), suggesting that they play an important function involved in catalysis. In particular, a Gly85Asp mutation in R2 is likely to affect binding of the SAM as it is adjacent to the GCGW motif, which has been shown to make contacts with the cofactor (Boissier *et al*., 2006). R7 and R12 each possess independent deletions of a single nucleotide, generating a frameshift and thus producing a premature stop codon in the *mmaA4* open

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd,

```
                                                                     F(R10)              T(R6)
  1 MTRMAEKPIS PTKTRTRFED IQAHYDVSDD FFALFQDPTR TYSCAYFEPP ELTLEEAQYA
                                  D(R2)      STOP(R7)
 61 KVDLNLDKLD LKPGMTLLDI GCGWGTTMRR AVERLDVNVI GLTLSKNQHA RCEQVLASID
121 TNRSRQVLLQ GWEDFAEPVD RIVSIEAFEH FGHENYDDFF KRCFNIMPAD GRMTVQSSVS STOP(R12)
181 YHPYEMAARG KKLSFETARF IKFIVTEIFP GGRLPSTEMM VEHGEKAGFT VPEPLSLRPH
241 YIKTLRIWGD TLQSNKDKAI EVTSEEVYNR YMKYLRGCEH YFTDEMLDCS LVTYLKPGAA
301 A
```

Fig. 4. Sequence of the *M. bovis* BCG *mmaA4* gene. The SAM binding site is underlined. The single amino acid changes in the three independent missense mutants reported in this study are indicated. Arrows indicate sites of stop codons introduced in two other independent mutants.

reading frame (Fig. 4). Interestingly, in R12, the stop codon in MmaA4 protein sequence is rather distal, eliminating only 61 C-terminal amino acids. Sequence of *mmaA4* was unaltered in strains R4, R5, R8 and R11 that produced keto-mycolates (Table 2). Sequences of other CMAS-encoding genes such as *mmaA2*, *cmaA2* and *pcaA* were also analysed and found to be unaltered in these strains (data not shown). Presently, the exact site(s) of mutation in the TAC-resistant strains that are wild-type for *mmaA4* remains unknown.

Functional complementation of mmaA4 *mutants restores drug susceptibility*

The *mmaA4* mutant strains were transformed with an integrative plasmid pMV361 carrying wild-type *M. tb_mmaA4* gene expressed from the constitutive *hsp60* promoter. Mycolic acid profiles of the *mmaA4*-complemented strains clearly showed significant amounts of keto-mycolates, and, in fact, this led to a relative diminution in the synthesis of α-mycolates (Fig. 5). Likewise, a parental BCG strain transformed with the same plasmid also showed a change in the relative ratio of α- : keto-mycolic acids. Thus, introduction of a wild-type *mmaA4* gene functionally complemented the lack of keto-mycolates in all *mmaA4* mutants.

Importantly, all *mmaA4*-complemented strains were characterized by a complete reversion of susceptibility to TAC. Figure 6 shows a comparison of growth of different strains on media containing TAC or its highly effective chemical analogues, SRI-224, SRI-222 and SRI-2344 (Alahari *et al*., 2007). R2 and R7 were selected for this analysis as they are comparably TAC-resistant, but R2 is derived from the parental strain that overexpresses EthA, whereas R7 derives from a parental strain carrying the empty pMV261 (Table 2). Corresponding parental strains were included as controls. R2 and R7 were able to grow in the presence of high concentrations of all of these analogues. In both resistant strains, complementation with *mmaA4* was sufficient to restore susceptibility to all the drugs tested. The susceptibility of these strains to different drugs was also assessed by measuring the MICs. As shown in Table 3, complementation with *mmaA4* in both strains lowered the MIC values of TAC or SRI-224 to the parental level. Moreover, R2 and R7 TAC-resistant mutants did not show significant altered susceptibility to either INH or rifampicin, the latter being a drug that needs to permeate the cell wall to have a lethal action. This suggests that resistance to TAC in the *mmaA4* mutants is unlikely to be associated with altered cell wall permeability. Overall, these results unambiguously establish that the TAC/SRI-224-resistant phenotype in these strains is linked to the lack of an active MmaA4 enzyme.

Increased susceptibility to TAC in BCG strains overproducing MmaA4

The role of MmaA4 in activation of TAC was further investigated by generating BCG strains that would express

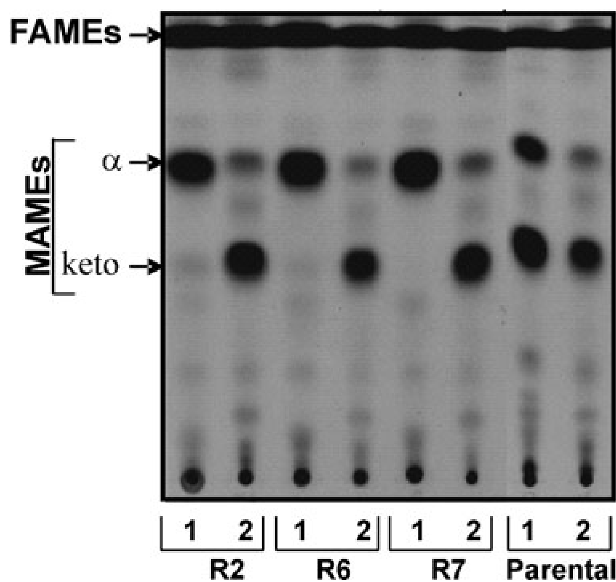

Fig. 5. Mycolic acid profile of various MmaA4-complemented strains. Autoradiogram showing radiolabelled mycolic acids from *mmaA4* mutant strains (R2 or R6 or R7) or parental *M. bovis* BCG strain (lanes 1) and corresponding strains overexpressing a functional *M. tb_mmaA4* (lanes 2). α- and keto-mycolates are indicated. The mutant strains lack keto-mycolates, which are regained by complementation with *mmaA4*.

© 2009 The Authors

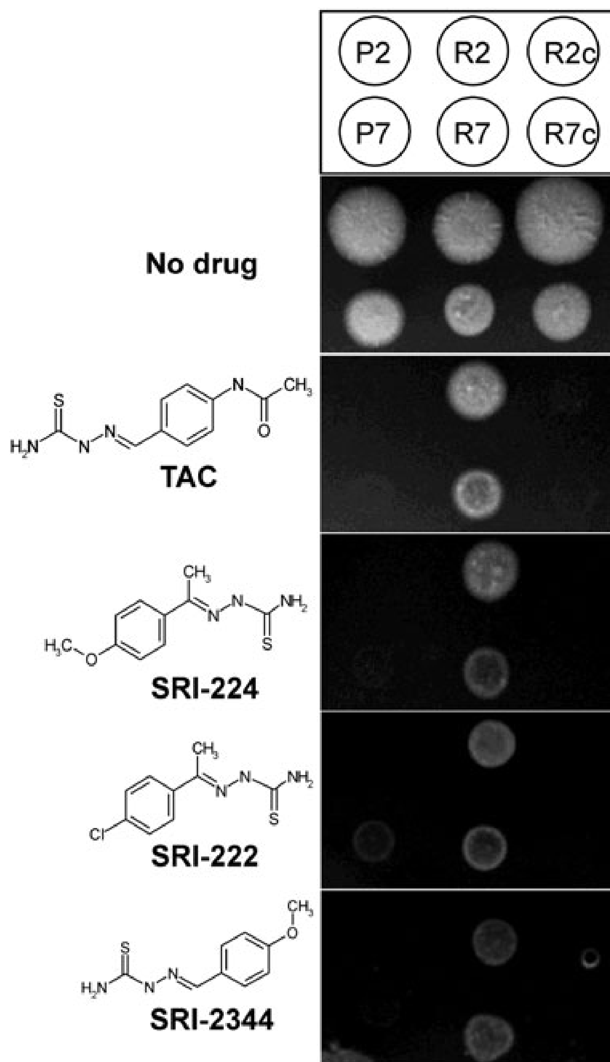

Fig. 6. Reversal to a drug susceptibility phenotype in mutants complemented with *M. tb_mmaA4*. Exponentially growing cultures of different strains were spotted on plates containing 10 μg ml^{-1} of either TAC or its chemical analogues as indicated. Schematic in the top panel depicts pattern of inoculation in the plates shown below. P2 corresponds to the parental strain (BCG/pMV261_*ethA*) of mutant R2; P7 represents the parental strain (BCG/pMV261) of mutant R7; R2 or R7 are TAC-resistant mutants; R2c or R7c correspond to the mutants complemented with *M. tb_mmaA4*. Chemical structure of the drug used are given on the left. SRI-222 and SRI-2344 are TAC analogues showing very high activity against H37Rv (Alahari *et al*., 2007). Only the mutant strains could grow at the high drug concentrations.

varying amounts of the protein. The *M. tb_mmaA4* gene was expressed from the constitutively, highly expressed *hsp60* promoter, either on a multicopy plasmid (pMV261) or on an integrative single copy plasmid (pMV361). Susceptibility of the strains to SRI-224 was assessed visually by growth inhibition on Middlebrook 7H11 plates supplemented with OADC and increasing drug concentrations. Figure 7A shows that both MmaA4-overproducing strains were more susceptible to growth inhibition by SRI-224 than the parental strain harbouring the empty pMV261. Identical results were obtained when SRI-224 was replaced by TAC (data not shown). This indicates that overexpression of MmaA4 confers increased susceptibility to these drugs and was further confirmed by the determination of the MICs of TAC and SRI-224 against these strains (Table 4). BCG carrying either pMV261- or pMV361-encoded *mmaA4* was up to five times more susceptible to either TAC or SRI-224 than the parental strain.

Given the very high similarity between *mmaA4* and *mmaA3* and the fact that these two genes are located adjacent in the *M. tb* genome (Yuan and Barry, 1996; Cole *et al*., 1998), we investigated whether overexpression of *mmaA3* would also affect susceptibility to TAC and SRI-224. In contrast to *mmaA4*, *mmaA3* expressed from either integrative or multicopy plasmids did not significantly alter drug susceptibility in the BCG strains (Table 4). Therefore, the *mmaA4*-overexpressing strains were more susceptible to TAC and SRI-224, but those overexpressing *mmaA3* were only as susceptible as the parental strain.

BCG strains differ in their abilities to produce methoxymycolic acids (Minnikin *et al*., 1983) and presence of methoxy-mycolic acids depends on MmaA3 which catalyses the *O*-methylation of hydroxymycolic acids precursors, generated by the action of MmaA4 (Yuan *et al*., 1998) (Fig. 1). The BCG strain Pasteur used in this study is a natural *mmaA3* mutant that fails to synthesize methoxymycolates (Behr *et al*., 2000). Hence, the BCG *mmaA4* mutants described here were also naturally mutated in *mmaA3*. As reported earlier (Yuan *et al*., 1998), the mycolic acid content of BCG strains overexpressing *M. tb_mmaA3* revealed the presence of methoxy-mycolates (data not shown). Unexpectedly, the BCG strain carrying pMV361_mmaA4 produced detectable amounts of

Table 3. MICs of various drugs against *M. bovis* BCG *mmaA4* mutants and *mmaA4*-complemented strains.

M. bovis BCG strain	TAC	SRI-224	INH	RIF
Parental strain (pMV261)	0.25	0.25	0.05–0.1	0.1
R7	10–25	5	0.05–0.1	0.05
R7 (361_mmaA4)	0.25	0.25	0.1	0.05
Parental strain (pMV261_ethA)	0.25	0.25	0.1	0.1
R2	10–25	10	0.05	0.1
R2 (361_mmaA4)	0.25	0.25	0.1	0.1

© 2009 The Authors

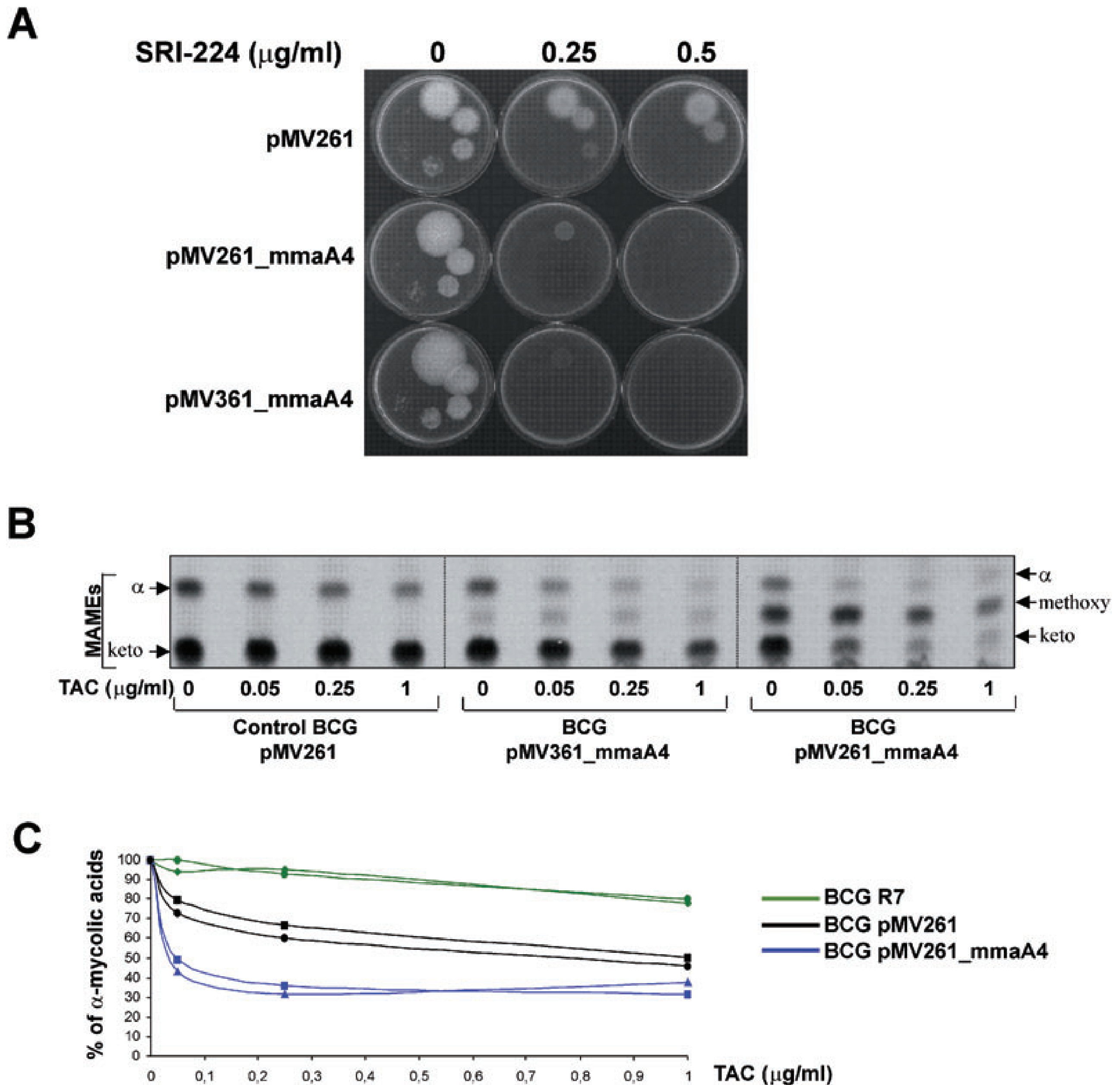

Fig. 7. Effect of MmaA4 overexpression on drug susceptibility and on mycolic acid profile in *M. bovis* BCG.
A. Antimycobacterial effect of SRI-224 against parental strain of *M. bovis* BCG (pMV261) and two strains overexpressing MmaA4 either on a multicopy vector (pMV261_*mmaA4*) or on an integrative construct (pMV361_*mmaA4*). Susceptibility of the strains was determined by growth on solid medium containing increasing concentrations of SRI-224. Serial 10-fold dilutions of actively growing culture were spotted and incubated at 37°C for 10–14 days.
B. Effect of MmaA4 overexpression on mycolic acid inhibition in *M. bovis* BCG harbouring either pMV261, pMV61_*mmaA4* or pMV361_*mmaA4* treated with low concentrations of TAC. Autoradiograph of MAMEs extracted from cultures metabolically radiolabelled with ^{14}C-acetate. Following extraction, MAMEs were resolved by TLC. Positions of α-, keto- and methoxy-mycolic acids are indicated.
C. Quantification of radiolabelled α-mycolates. Silica from TLC plate corresponding to region of localization of α-mycolates was scraped off and counted. For each strain, the value obtained for untreated sample was taken as 100%. Results from two independent experiments are presented.

methoxy-mycolic acids and this phenomenon was even more pronounced in the strain harbouring pMV261_ *mmaA4* (Fig. 7B). These results indicate that by providing sufficient levels of hydroxymycolic acids, which are substrates of MmaA3, BCG Pasteur strain regains an ability to produce methoxy-mycolates. Together, these results also indicate that it is not simply the presence of methoxy-mycolic acids that alters susceptibility to the drugs.

© 2009 The Authors

Table 4. MICs of TAC and SRI-224 against *M. bovis* BCG strains overexpressing the *M. tuberculosis mmaA4* or *mmaA3* genes.

M. bovis BCG strain	TAC	SRI-224
Parental strain (pMV261)	0.25	0.25
pMV261_mmaA4	0.1	0.1
pMV361_mmaA4	0.05	0.1
pMV261_mmaA3	0.25	0.25–1
pMV361_mmaA3	0.25	0.25–1

We next investigated the effect of low doses of TAC on the mycolic acid profile in the parental strain and the strains that differentially overexpress *mmaA4*. TLC analysis (Fig. 7B) indicates that the higher the expression of *mmaA4*, the more severe were the effects on mycolic acid synthesis (α-mycolates and keto-mycolates). Figure 7C depicts quantification of radiolabelled α-mycolic acids that were recovered from TLC plates and estimated by scintillation counting. Again, the selected strains showed a positive correlation between levels of MmaA4 expression and susceptibility to the drug: R7, which lacks *mmaA4*, had the lowest susceptibility and could retain high amounts of α-mycolates despite treatment with TAC, while the *mmaA4*-overexpressing strain was highly susceptible and synthesized about three times lower amounts of α-mycolates under the same conditions of growth. These results suggest that, at sublethal drug concentrations, inhibition of α-mycolic acid cyclopropanation (as a consequence of CMAS inhibition) is dependent on expression levels of both EthA and MmaA4.

Disruption of mmaA4 *in* M. tuberculosis *leads to high resistance levels to TAC*

Whether the correlation between increased resistance to TAC and SRI-224 and mutations in *mmaA4* is restricted to BCG or is a more general mechanism was investigated through the use of defined *mmaA3* and *mmaA4* mutants in *M. tb* CDC1551. These mutants contain a *Himar1* transposon at position 459 in *mmaA3* (strain JHU0643c-459) and 583 in *mmaA4* (strain JHU0642c-583) which disrupted the genes (Lamichhane *et al.*, 2003) (Table 1). The phenotype of *mmaA4*::Tn strain was confirmed by analysis of the mycolic acid profile, which showed lack of oxygenated mycolic acids (Fig. S1). In addition, introduction of a functional copy of *mmaA*4 in this mutant allowed a complete restoration of the mycolic acid profile (Fig. S1). Resistance to TAC was then compared between *M. tb* strain CDC1551, the *mmaA3* and *mmaA4* null mutants and the *mmaA4* mutant complemented with the wild-type *mmaA4* gene carried on the single copy plasmid pMV361 (Table 5). The *mmaA4* mutant showed two orders of magnitude higher resistance to TAC and to SRI-224, while complementation with a functional *mmaA4* gene restored the wild-type phenotype. However, *mmaA3* mutation did not confer resistance to TAC. Moreover, resistance to INH in all the above strains was comparable (data not shown). Thus, in agreement with the data obtained for BCG, optimal activity of TAC against *M. tb* requires a functional MmaA4.

Discussion

Mutations that confer resistance to a drug are often due to a lack of activation of the drug or due to a resulting modification in the molecular target of the drug. In a screen for spontaneous mutants resistant to TAC, *mmaA4* was uncovered. While other members of the CMAS family of mycolic acid methyltransferases are inhibited by TAC, MmaA4 is not inhibited by this drug (Alahari *et al.*, 2007). Hence, if mutations in *mmaA4* confer TAC resistance, it is logical to conclude that MmaA4 is involved in activation of TAC. Notably, MmaA4 is functionally distinct from other members of the CMAS family, in that, in addition to introducing a methyl branch on the meromycolic acid, it also introduces a hydroxyl group (Boissier *et al.*, 2006) (Fig. 1). Whether it is the latter activity or an as yet unidentified activity of MmaA4 that allows it to contribute to activation of TAC or SRI-224 is debatable. The notion that MmaA4 is required for activation of the pro-drug is also supported by the fact that overexpression of a functional *mmaA4* increases the susceptibility to TAC and SRI-224, a feature that has been observed for other mycobacterial pro-drug processes (Zhang *et al.*, 1993; Scorpio and Zhang, 1996; Baulard *et al.*, 2000; DeBarber *et al.*, 2000). Decreased permeability to TAC due to changes in mycolic acid composition could be a possible reason for the resistance in *mmaA4* mutants. Although we have not carried out detailed studies on permeability, our data argue against this possibility because susceptibility to INH and RIF remained unchanged, and also because exposure to TAC led to the expected diminution in α-mycolates in these mutants.

Enzymes that serve to activate commonly used antitubercular drugs are usually non-essential enzymes such as the catalase-peroxydase KatG, activator of INH (Zhang *et al.*, 1992), the pyrazinamidase PncA, activator of

Table 5. MICs of TAC and SRI-224 *M. tuberculosis* CDC1551 *mmaA3* and *mmaA4* mutants.

M. tuberculosis CDC1551	TAC	SRI-224
Wild type	0.1	0.2
mmaA3::Tn	0.05	nd
mmaA4::Tn	10	20
mmaA4::Tn complemented	0.1	1.25

nd, not determined.

© 2009 The Authors

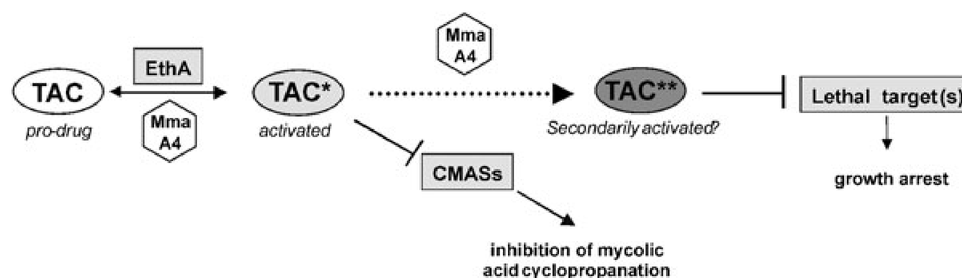

Fig. 8. Schematic showing dual processing of TAC by EthA and MmaA4. TAC is a pro-drug which is activated to TAC* by the action of the primary activator EthA. TAC* is necessary and sufficient for inhibiting cyclopropanation of mycolic acids by CMAS enzymes, but does not inhibit MmaA4. It is possible that MmaA4 plays a supplementary role in inhibition of the other CMAS enzymes. Inhibition of the lethal target(s) may be brought about by a secondary activated form, TAC**. Generation of TAC** requires MmaA4. TAC** may be produced by direct/indirect action of MmaA4 or it may be a secondary, activated form produced by EthA in the presence of MmaA4 (dotted line).

pyrazinamide (Scorpio and Zhang, 1996), the glucose 6-phosphate dehydrogenase FGD1 (Stover *et al.*, 2000) for PA-824, or the monoxygenase EthA, activator of ETH, TAC and isoxyl (Baulard *et al.*, 2000; DeBarber *et al.*, 2000). Resistance to PA-824 is mediated by loss of a specific glucose 6-phosphate dehydrogenase FGD1 (Bashiri *et al.*, 2008) or its deazaflavin cofactor F420 (Stover *et al.*, 2000). Apart from these two components, an accessory protein, Rv3547, with no known function, is also necessary for PA-824 susceptibility (Manjunatha *et al.*, 2006). Recently, activation of ETH was shown to require mycothiol biosynthesis in addition to activation by EthA (Vilcheze *et al.*, 2008). Thus, there is an emerging concept in the understanding of antimycobacterial drug action that there is more than one player in the activation of a drug and/or existence of more than one activated forms of the drug that likely target different essential processes. The present study also agrees with this new concept by identifying MmaA4 as a key player, along with EthA, in the activation of TAC.

Mycobacterium tuberculosis mutants in *ethA* are cross-resistant to ETH, TAC and isoxyl (DeBarber *et al.*, 2000), while overexpression of this activator results in higher susceptibility to these drugs (Baulard *et al.*, 2000; Dover *et al.*, 2007). Loss of a functional EthA does not affect growth *in vitro*, indicating that EthA is not the target of these drugs. Similarly, data presented here establish that MmaA4 is likely required for activation of the drug. MmaA4 mutants are TAC-resistant and the level of *mmaA4* expression determines the drug susceptibility. MmaA4 is not the target of TAC since it has been shown to be unaffected even at high concentrations of TAC, in sharp contrast to the other CMAS enzymes (Alahari *et al.*, 2007). However, it is likely required for activation of the drug. Based on these data, a model is proposed (Fig. 8). In strains carrying an active EthA, susceptibility to TAC appears to depend on level of intracellular MmaA4. TAC is a pro-drug which is primarily activated by EthA to TAC*, which inhibits production of 'mature' mycolic acids by targeting methyltransferases of the CMAS family, such as PcaA, CmaA2 and MmaA2, but not MmaA4. However, the CMASs are not the essential lethal target of the drug (Glickman *et al.*, 2000; Glickman, 2003; Alahari *et al.*, 2007). Primary activation by EthA is necessary, but not sufficient to hit the lethal target(s). TAC may be secondarily modified to its lethal form, TAC**, ultimately causing growth arrest. Given that in strains overexpressing MmaA4, the lethal effects of TAC are enhanced, it is possible that MmaA4 directly interacts with TAC or with EthA to produce TAC**. Alternatively, generation of TAC** may be independent of EthA, while likely requiring MmaA4 to interact with other, unidentified partners. At this point, existence of the activated forms of TAC and identity of the lethal target(s) remain to be demonstrated.

The likely interaction between TAC and MmaA4 was experimentally tested with the aim of uncovering TAC**. Since MmaA4 is a methyltransferase, methylation of TAC was investigated. BCG strains were allowed to react with TAC in the presence of [^{14}C-methyl]-methionine, which served as a methyl donor to *de novo* methylation. Mycobacterial cell lysates were collected at various time points and the different forms of TAC were resolved by TLC/autoradiography (Fig. S2). While methylated forms of TAC were detected, these were present in both the *mmaA4* mutant and its corresponding complemented strain. This clearly indicates that TAC is metabolized *in vivo* and that methylation of the drug occurs although this process appears independent of MmaA4. Moreover, it remains unknown whether these methylated forms represent the active lethal products of TAC and whether these are a result of action of EthA or of other unknown metabolic processes.

Unravelling the mechanism of involvement of MmaA4 in drug activation and the mode of action of TAC may also reveal new aspects of mycolic acid synthesis hitherto unknown. Supporting this view, we have identified a new population of mycolic acids that specifically accumulates in drug-treated MmaA4 mutants (Fig. 3A), and whose

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd,

structures remain to be determined. This highlights the complexity of mycolic acid biosynthesis and the extraordinary capacity of mycobacteria to increase the diversity of their cell wall lipids.

Intriguingly, none of the TAC-resistant strains isolated here, especially those originating from BCG/pMV261 parent, had mutations in *ethA*, which would have been expected as clinical strains co-resistant to ETH, isoxyl and TAC are often mutated in *ethA* (DeBarber *et al*., 2000), and conversely, the majority of TAC-resistant mutants described here were affected in *mmaA4*. One possible explanation why *mmaA4* has never been uncovered in past studies based on sequencing resistant clinical isolates could be due to the fact that *mmaA4* knockout strains of *M. tb* are highly attenuated *in vivo* (Dubnau *et al*., 2000), and as a consequence, those mutants are very likely to be lost. A recent study demonstrated that mycolic acid modification by MmaA4 renders *M. tb* capable of repressing IL-12 production, thereby assisting the bacterium in establishing chronic infection, since the attenuation depends on IL12p40-mediated immunity (Dao *et al*., 2008). Thus, due to its highly attenuated phenotype, one can presume that *mmaA4* mutants which would be TAC-resistant are highly unlikely to be found among clinical isolates. Among the members of our collection, four strains with no mutations in *mmaA4* or *ethA* were found to exhibit various levels of resistance to TAC. R8 is particularly interesting since it is highly resistant to the drug and presents a mycolic acid profile similar to the parental strain, and hence may be mutated in the lethal target of TAC. Work is now in progress to characterize the remaining TAC-resistant strains isolated in the course of this study and to identify the target(s) of the drug.

Mycolic acids in *M. tb* are present as a mixture of various related molecules with different chemical groups at the proximal and distal positions of their meromycolic acid chain (Fig. 1). These discrete chemical variations are of crucial biological importance since it has been shown that mutation resulting in the loss of these chemical functions, and particularly the keto and methoxy groups profoundly affect the permeability of the cell envelope to solutes and severely affect virulence and pathogenicity of the mutant strains (Yuan *et al*., 1998; Dubnau *et al*., 2000; Glickman *et al*., 2000; Rao *et al*., 2005; 2006). Out of the eight mycolic acid SAM-dependent methyltransferases encoded in *M. tb*, only MmaA4 (also known as Hma) has been shown to be necessary and sufficient for the synthesis of oxygenated mycolic acids. It is very intriguing that, despite their structural relatedness (Huang *et al*., 2002; Boissier *et al*., 2006), some of these methyltransferases (MmaA2, CmaA2 and PcaA) are inhibited by TAC, whereas MmaA4 appears necessary to activate the drug. Obviously, co-crystallization studies of CMAs or MmaA4 with activated TAC are required to explain how subtle structural specificities can govern these major differences with respect to TAC.

This study represents the first description of involvement in drug activation of an enzyme like MmaA4, which is also required for synthesis of mycolic acids, virulence and participates in immune evasion mechanisms in *M. tb* (Dubnau *et al*., 2000; Dao *et al*., 2008). Previous studies on transcriptional profiling of *M. tb* in the lungs of infected mice have shown that the *mmaA4* gene is upregulated *in vivo*, compared with its level in bacteria growing in culture (Rachman *et al*., 2006). This is an interesting observation, which opens the possibility that TAC may be more efficient *in vivo* than in *in vitro* growing cultures.

Experimental procedures

Cultural conditions and mutagenesis

Mycobacterium bovis BCG strain Pasteur 1173P2, referred to as BCG, or *M. tb* strain H37Rv were routinely maintained in Sauton's medium at 37°C. Mycobacterial strains were transformed and selected on Middlebrook 7H11 agar supplemented with oleic-albumin-dextrose-catalase enrichment (OADC, Difco), containing kanamycin or hygromycin at 25 or 50 μg ml^{-1}, respectively, as required. Spontaneous mutants, resistant to high levels of either TAC or SRI-224, were selected as follows: BCG/pMV261 or BCG/pMV261-*ethA*, which served as parental strains, were grown to exponential phase, diluted and plated at approximately 1×10^8 cells ml^{-1} on 7H11 agar medium containing either 1.25 or 2.5 or 10 μg ml^{-1} TAC or SRI-224. Plates were incubated at 37°C up to 4 weeks. Single colonies were picked and passaged three times through Sauton's medium containing 10 μg ml^{-1} TAC or SRI-224 before maintaining them on regular medium without drugs.

Plasmids and DNA manipulation

Restriction enzymes, T4 DNA ligase and Vent DNA polymerase were purchased from New England Biolabs. The *Escherichia coli*-mycobacterial shuttle vector pMV261 containing the *hsp60* promoter was used as described previously (Stover *et al*., 1991). The *mmaA3* or *mmaA4* coding sequences were amplified from *M. tb* genomic DNA using primers, which were adapted for cloning in the MscI and either EcoRI or HindIII sites, respectively, of pMV261 (Table 1). The inserts were then subcloned from pMV261 into pMV361 using the XmnI and HindIII sites present in both the vectors. The integrity of all of these constructs was verified by nucleotide sequencing.

Sequence of the different CMAS alleles: *mmaA2*, *mmaA3*, *mmaA4*, *cmaA2* or *pcaA* in the various BCG strains was obtained from PCR amplification of the concerned genomic region from cell suspensions. Sequence of primers used in this study are given in Table S1. For the purpose of PCR amplification of genomic regions using cell suspensions, three independent colonies were randomly chosen and allowed to grow in Sauton's medium supplemented with the appropriate antibiotic. Cell pellets from 1 ml of each culture

© 2009 The Authors

were re-suspended in 100 μl of sterile water and held at 95°C for 5 min. An aliquot of 2 μl of these cell lysates served as a template to PCR amplify the desired region with appropriate primers. Vent DNA polymerase was employed in a two-step cycle that included denaturation at 95°C and a combined step of annealing and extension at 68°C for 1 min. After 30 cycles, a supplementary extension at 72°C for 10 min was provided. The PCR products were sequenced using the same primers to obtain sequence of both the DNA strands of the coding region, along with up to 150 bp of upstream sequences.

Lipid analysis

Extraction of cell wall mycolic acids from mycobacterial cells was carried out as previously described (Dover *et al.*, 2007). Briefly, cell pellets were washed and treated with tetrabutyl ammonium hydroxide (TBAH) at 100°C overnight. Mycolic acids were methyl-esterified and extracted in diethyl ether. Extracts were dried and re-suspended in dichloromethane for application to a silica-coated plate for TLC. Mycolates were resolved on normal-phase TLC in petroleum ether/acetone (19:1, v/v), run three times. In case of silica plates impregnated with 10% silver nitrate, petroleum ether/diethyl ether (17/3, v/v) was used for three runs. Two-dimensional TLC on silver nitrate impregnated plates was carried out with hexane/ethyl acetate (19/1, v/v), twice in the first dimension, followed by petroleum ether/diethyl ether (17/3, v/v) three times in the second dimension. Lipids were visualized by spraying the TLC plate with 5% molybdophosphoric acid (MPA) in ethanol followed by charring with a heat gun. Different subtypes of mycolic acids were resolved in petroleum ether/acetone (19/1, v/v) on a preparative TLC, scraped off the TLC plate separately and extracted in diethyl ether to obtain purified samples that could be analysed by NMR or MS. For metabolic radiolabelling of lipids, 1 μCi ml^{-1} [2-^{14}C]acetate (56 mCi $mmol^{-1}$, Amersham Biosciences) was added to the growth medium for 6–8 h, followed by harvesting of the cells and extraction of mycolates as described above. For visualizing drug-induced changes in the mycolates profile, 10 μg ml^{-1} TAC or SRI-224 was added to the growth medium overnight followed by metabolic radiolabelling the next day. Autoradiograms were obtained by overnight exposure to Kodak Biomax MR film to reveal [^{14}C]-labelled lipids.

Drug susceptibility testing

Susceptibility of *M. bovis* BCG strains to various inhibitors was determined on Middlebrook 7H11 solid medium containing OADC enrichment with increasing drug concentrations as reported earlier (Dover *et al.*, 2007). Serial 10-fold dilutions of each actively growing culture were plated and incubated at 37°C for 2 weeks. The MIC was defined as the minimum concentration required to inhibit 99% of the growth. Susceptibility of *M. tb* CDC1551 strains to various inhibitors was determined on Middlebrook 7H9 medium containing 10% OADC with twofold dilutions of each drug (TAC or SRI-224). Each tube was inoculated with 10^5 actively growing bacilli of each strain and incubated at 37°C without shaking for 2 weeks. Growth was visually analysed at days 7 and 14. MIC value was the lowest drug concentration that completely inhibited growth.

Assay for in vitro *methylation of drugs*

Methylated forms of TAC or SRI-224 were identified as follows. Exponentially growing cells of various BCG strains, at OD_{600} of approximately 1.0, were concentrated at least 10-fold and re-suspended in phosphate buffer. Cell suspension was held at 37°C and supplied ^{14}C-methyl-methionine, either in the presence or in the absence of 100 μg ml^{-1} of either drug for various lengths of time as desired. Total volume of this reaction mix was 0.5 ml. Cells, along with the above reaction mix, were lysed using 0.5 ml of glass beads in a beadbeater operated at 30 s^{-1} for 10 min and maintained on ice subsequently. Drugs were extracted in 0.5 ml of ethyl acetate. Aqueous and organic phases were separated by high-speed centrifugation and the top organic layer was recovered. Latter was dried under nitrogen and re-suspended in 50 μl of ethyl acetate. Typically, 20 μl of the extract was applied to silica-coated TLC plate. Extracts were resolved in toluene/ethyl acetate (7:3, v/v) for SRI-224 or in ethyl acetate/ethanol (19/1, v/v) for TAC. TLC plate was stained with MPA as described above for mycolates. Methylated forms were visualized by exposure to an X-ray film for the required duration.

Chemical analyses

Mycolic acid methyl esters used for structural analyses were isolated by preparative TLC as described above and used as previously published (Alahari *et al.*, 2007). MS analyses of mycolic methyl esters were performed on a Voyager Elite reflectron MALDI-TOF mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA), equipped with a 337 nm UV laser. Samples were solubilized in 1 μl of chloroform/methanol (2:1) and mixed on target with 1 μl of 2,5-dihydroxybenzoic acid matrix solution (10 mg ml^{-1} dissolved in chloroform/methanol 2:1). For NMR, samples were dissolved in 300 μl of deuterated chloroform and transferred into Shigemi tubes matched for D_2O. One hundred microlitres of deuterium oxide was added to avoid solvent evaporation. Unidimensional proton spectra were recorded at 299 K on a 400 MHz Avance Bruker spectrometer equipped with a broad-band inverse probe. Proton chemical shifts were referenced to TMS or residual protonated chloroform signal. Previously published proton assignment was used (Alahari *et al.*, 2007).

Acknowledgements

Authors wish to thank Eric Rubin and Chris Sassetti for helpful discussions. L.K. is supported by a Grant from the Centre National de la Recherche Scientifique (CNRS) (Action Thématique Incitative sur Programme 'Microbiologie Fondamentale'). The support of NIH Grants AI43846 and AI30036 to W.R.B. is gratefully acknowledged.

References

Alahari, A., Trivelli, X., Guerardel, Y., Dover, L.G., Besra, G.S., Sacchettini, J.C., *et al.* (2007) Thiacetazone, an

© 2009 The Authors

antitubercular drug that inhibits cyclopropanation of cell wall mycolic acids in mycobacteria. *PLoS ONE* **2:** e1343.

Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K.S., Wilson, T., *et al.* (1994) *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263:** 227–230.

Bashiri, G., Squire, C.J., Moreland, N.J., and Baker, E.N. (2008) Crystal structures of an F420-dependent glucose-6-phosphate dehydrogenase FGD1 involved in the activation of the anti-TB drug candidate PA-824 reveal the basis of coenzyme and substrate binding. *J Biol Chem* **283:** 17531–17541.

Baulard, A.R., Betts, J.C., Engohang-Ndong, J., Quan, S., McAdam, R.A., Brennan, P.J., *et al.* (2000) Activation of the pro-drug ethionamide is regulated in mycobacteria. *J Biol Chem* **275:** 28326–28331.

Behr, M.A., Schroeder, B.G., Brinkman, J.N., Slayden, R.A., and Barry, C.E., 3rd (2000) A point mutation in the *mma3* gene is responsible for impaired methoxymycolic acid production in *Mycobacterium bovis* BCG strains obtained after 1927. *J Bacteriol* **182:** 3394–3399.

Bermudez, L.E., Reynolds, R., Kolonoski, P., Aralar, P., Inderlied, C.B., and Young, L.S. (2003) Thiosemicarbazole (thiacetazone-like) compound with activity against *Mycobacterium avium* in mice. *Antimicrob Agents Chemother* **47:** 2685–2687.

Boissier, F., Bardou, F., Guillet, V., Uttenweiler-Joseph, S., Daffe, M., Quemard, A., and Mourey, L. (2006) Further insight into *S*-adenosylmethionine-dependent methyltransferases: structural characterization of Hma, an enzyme essential for the biosynthesis of oxygenated mycolic acids in *Mycobacterium tuberculosis*. *J Biol Chem* **281:** 4434–4445.

Brennan, P.J., and Nikaido, H. (1995) The envelope of mycobacteria. *Annu Rev Biochem* **64:** 29–63.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., *et al.* (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537–544.

Daffe, M., and Draper, P. (1998) The envelope layers of mycobacteria with reference to their pathogenicity. *Adv Microb Physiol* **39:** 131–203.

Dao, D.N., Sweeney, K., Hsu, T., Gurcha, S.S., Nascimento, I.P., Roshevsky, D., *et al.* (2008) Mycolic acid modification by the *mmaA4* gene of *M. tuberculosis* modulates IL-12 production. *PLoS Pathog* **4:** e1000081.

Davidson, P.T., and Le, H.Q. (1992) Drug treatment of tuberculosis – 1992. *Drugs* **43:** 651–673.

DeBarber, A.E., Mdluli, K., Bosman, M., Bekker, L.G., and Barry, C.E., 3rd (2000) Ethionamide activation and sensitivity in multidrug-resistant *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **97:** 9677–9682.

Dinadayala, P., Laval, F., Raynaud, C., Lemassu, A., Laneelle, M.A., Laneelle, G., and Daffe, M. (2003) Tracking the putative biosynthetic precursors of oxygenated mycolates of *Mycobacterium tuberculosis*. Structural analysis of fatty acids of a mutant strain devoid of methoxy- and ketomycolates. *J Biol Chem* **278:** 7310–7319.

Dover, L.G., Alahari, A., Gratraud, P., Gomes, J.M., Bhowruth, V., Reynolds, R.C., *et al.* (2007) EthA, a common activator of thiocarbamide-containing drugs acting on different mycobacterial targets. *Antimicrob Agents Chemother* **51:** 1055–1063.

Dubnau, E., Chan, J., Raynaud, C., Mohan, V.P., Laneelle, M.A., Yu, K., *et al.* (2000) Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. *Mol Microbiol* **36:** 630–637.

Glickman, M.S. (2003) The *mmaA2* gene of *Mycobacterium tuberculosis* encodes the distal cyclopropane synthase of the alpha-mycolic acid. *J Biol Chem* **278:** 7844–7849.

Glickman, M.S., Cox, J.S., and Jacobs, W.R., Jr. (2000) A novel mycolic acid cyclopropane synthetase is required for cording, persistence, and virulence of *Mycobacterium tuberculosis*. *Mol Cell* **5:** 717–727.

Huang, C.C., Smith, C.V., Glickman, M.S., Jacobs, W.R., Jr, and Sacchettini, J.C. (2002) Crystal structures of mycolic acid cyclopropane synthases from *Mycobacterium tuberculosis*. *J Biol Chem* **277:** 11559–11569.

Kremer, L., Baulard, A.R., and Besra, G.S. (2000) Genetics of mycolic acid biosynthesis. In *Molecular Genetics of Mycobacteria*. Hatfull, G.F., and Jacobs, W.R., Jr (eds). Washington, DC: American Society for Microbiology Press, pp. 173–190.

Kremer, L., Guerardel, Y., Gurcha, S.S., Locht, C., and Besra, G.S. (2002) Temperature-induced changes in the cell-wall components of *Mycobacterium thermoresistibile*. *Microbiology* **148:** 3145–3154.

Lamichhane, G., Zignol, M., Blades, N.J., Geiman, D.E., Dougherty, A., Grosset, J., *et al.* (2003) A postgenomic method for predicting essential genes at subsaturation levels of mutagenesis: application to *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **100:** 7213–7218.

Laval, F., Laneelle, M.A., Deon, C., Monsarrat, B., and Daffe, M. (2001) Accurate molecular mass determination of mycolic acids by MALDI-TOF mass spectrometry. *Anal Chem* **73:** 4537–4544.

Manjunatha, U.H., Boshoff, H., Dowd, C.S., Zhang, L., Albert, T.J., Norton, J.E., *et al.* (2006) Identification of a nitroimidazo-oxazine-specific protein involved in PA-824 resistance in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **103:** 431–436.

Minnikin, D.E., Minnikin, S.M., Dobson, G., Goodfellow, M., Portaels, F., van den Breen, L., and Sesardic, D. (1983) Mycolic acid patterns of four vaccine strains of *Mycobacterium bovis* BCG. *J Gen Microbiol* **129:** 889–891.

Nunn, P., Porter, J., and Winstanley, P. (1993) Thiacetazone – avoid like poison or use with care? *Trans R Soc Trop Med Hyg* **87:** 578–582.

Rachman, H., Strong, M., Ulrichs, T., Grode, L., Schuchhardt, J., Mollenkopf, H., *et al.* (2006) Unique transcriptome signature of *Mycobacterium tuberculosis* in pulmonary tuberculosis. *Infect Immun* **74:** 1233–1242.

Rao, V., Fujiwara, N., Porcelli, S.A., and Glickman, M.S. (2005) *Mycobacterium tuberculosis* controls host innate immune activation through cyclopropane modification of a glycolipid effector molecule. *J Exp Med* **201:** 535–543.

Rao, V., Gao, F., Chen, B., Jacobs, W.R., Jr, and Glickman, M.S. (2006) *Trans*-cyclopropanation of mycolic acids on trehalose dimycolate suppresses *Mycobacterium tuberculosis*-induced inflammation and virulence. *J Clin Invest* **116:** 1660–1667.

© 2009 The Authors
Journal compilation © 2009 Blackwell Publishing Ltd,

Scorpio, A., and Zhang, Y. (1996) Mutations in *pncA*, a gene encoding pyrazinamidase/nicotinamidase, cause resistance to the antituberculous drug pyrazinamide in tubercle bacillus. *Nat Med* **2:** 662–667.

Stover, C.K., de la Cruz, V.F., Fuerst, T.R., Burlein, J.E., Benson, L.A., Bennett, L.T., *et al.* (1991) New use of BCG for recombinant vaccines. *Nature* **351:** 456–460.

Stover, C.K., Warrener, P., VanDevanter, D.R., Sherman, D.R., Arain, T.M., Langhorne, M.H., *et al.* (2000) A small-molecule nitroimidazopyran drug candidate for the treatment of tuberculosis. *Nature* **405:** 962–966.

Takayama, K., Wang, C., and Besra, G.S. (2005) Pathway to synthesis and processing of mycolic acids in *Mycobacterium tuberculosis*. *Clin Microbiol Rev* **18:** 81–101.

Vilcheze, C., Wang, F., Arai, M., Hazbon, M.H., Colangeli, R., Kremer, L., *et al.* (2006) Transfer of a point mutation in *Mycobacterium tuberculosis inhA* resolves the target of isoniazid. *Nat Med* **12:** 1027–1029.

Vilcheze, C., Av-Gay, Y., Attarian, R., Liu, Z., Hazbon, M.H., Colangeli, R., *et al.* (2008) Mycothiol biosynthesis is essential for ethionamide susceptibility in *Mycobacterium tuberculosis*. *Mol Microbiol* **69:** 1316–1329.

Wang, F., Langley, R., Gulten, G., Dover, L.G., Besra, G.S., Jacobs, W.R., Jr, and Sacchettini, J.C. (2007) Mechanism of thioamide drug action against tuberculosis and leprosy. *J Exp Med* **204:** 73–78.

Yuan, Y., and Barry, C.E., 3rd (1996) A common mechanism for the biosynthesis of methoxy and cyclopropyl mycolic acids in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **93:** 12828–12833.

Yuan, Y., Zhu, Y., Crane, D.D., and Barry, C.E., 3rd (1998) The effect of oxygenated mycolic acid composition on cell wall function and macrophage growth in *Mycobacterium tuberculosis*. *Mol Microbiol* **29:** 1449–1458.

Zhang, Y., Heym, B., Allen, B., Young, D., and Cole, S. (1992) The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358:** 591–593.

Zhang, Y., Garbe, T., and Young, D. (1993) Transformation with katG restores isoniazid-sensitivity in *Mycobacterium tuberculosis* isolates resistant to a range of drug concentrations. *Mol Microbiol* **8:** 521–524.

Supporting information

Additional supporting information may be found in the online version of this article.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

© 2009 The Authors