The *mabA* gene from the *inhA* operon of *Mycobacterium tuberculosis* encodes a 3-ketoacyl reductase that fails to confer isoniazid resistance

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A target of the anti-tuberculosis drugs isoniazid (INH) and ethionamide (ETH) has been shown to be an enoyl reductase, encoded by the *inhA* gene. The *mabA* (mycolic acid biosynthesis A) gene is located immediately upstream of *inhA* in *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium smegmatis*. The *MabA* protein from *M. tuberculosis* was expressed in *Escherichia coli* and shown to have 3-ketoacyl reductase activity, consistent with a role in mycolic acid biosynthesis. In *M. smegmatis*, *inhA* and *mabA* are independently transcribed, but in *M. tuberculosis* and *M. bovis* BCG, *mabA* and *inhA* constitute a single operon. Several INH-ETH-resistant *M. tuberculosis* clinical isolates contain point mutations in the ribosome-binding site of *mabA* in the *mabA*-*inhA* operon. However, genetic dissection of this operon reveals that the INH-ETH-resistance phenotype is encoded only by *inhA*, and not by *mabA*.

Keywords: *Mycobacterium tuberculosis*, isoniazid, ethionamide, drug resistance, *inhA* gene

INTRODUCTION

Tuberculosis is one of the world's most deadly infectious diseases (World Health Organization, 1997). The recent emergence of multidrug-resistant strains of *Mycobacterium tuberculosis* poses a serious threat to tuberculosis-control programs worldwide (Snider *et al.*, 1994). Understanding the mechanisms of resistance to the available antimycobacterial drugs and discovery of new drug targets are necessary for the treatment of tuberculosis.

The gene (*inhA*) encoding a target for isoniazid (INH) and its analogue, ethionamide (ETH), was cloned in

1994 (Banerjee *et al.*, 1994). Mutations in *inhA* were shown to confer resistance to both INH and ETH in drug-resistant *M. tuberculosis* clinical isolates (Heym *et al.*, 1994; Kapur *et al.*, 1996; Musser *et al.*, 1996; Ristow *et al.*, 1995; Rouse *et al.*, 1995; Telenti *et al.*, 1997; Victor *et al.*, 1997). KatG- (Zhang *et al.*, 1992) activated INH is proposed to inhibit the *inhA*-encoded enoyl reductase (ER) enzyme, which is involved in the biosynthesis of mycolic acids (long-chain fatty acids of mycobacteria) (Johnsson *et al.*, 1995; Wheeler & Anderson, 1996; Quemard *et al.*, 1995; Dessen *et al.*, 1995; Rozwarski *et al.*, 1998).

An ORF has been found immediately upstream of *inhA* in all the mycobacteria tested thus far (Banerjee *et al.*, 1994). This ORF, a strong homologue of many 3-ketoacyl reductases (KARs), was previously designated *orf1* and later renamed *mabA* (Musser *et al.*, 1996), due to its probable involvement in mycolic acid biosynthesis (Kikuchi *et al.*, 1989; Quemard *et al.*, 1995). *mabA* from *Mycobacterium smegmatis*, which is transcribed separately from *inhA*, has been shown not to mediate INH-ETH resistance. Despite the fact that, unlike in *M. smegmatis*, *mabA* constitutes an operon with *inhA* in

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Abbreviations: CAT, chloramphenicol acetyltransferase; ER, enoyl reductase; ETH, ethionamide; INH, isoniazid; KAR, 3-ketoacyl reductase.

The GenBank/EMBL accession numbers for the sequences reported in this paper are U66800 (*M. smegmatis mabA*) and U66801 (*M. tuberculosis mabA*). The *mabA* sequence from *M. bovis* BCG is identical to that of *M. tuberculosis*.

M. tuberculosis, it was not initially tested whether MabA from *M. tuberculosis* plays a role in mediating drug resistance. Based on the high (82% identity) homology between these two MabAs, it was assumed that *M. tuberculosis* MabA is not involved in INH resistance. However, later on, INH-resistant clinical isolates of *M. tuberculosis* were frequently reported to have mutations in the ribosome-binding site (RBS) of *mabA* (Kapur *et al.*, 1996; Musser *et al.*, 1996; Rouse *et al.*, 1995; Telenti *et al.*, 1997; Victor *et al.*, 1997). This observation supported the speculation that, in *M. tuberculosis*, MabA could be an additional target for INH and ETH, and overexpression of MabA could mediate INH resistance by virtue of increased drug titration (Musser, 1995). Hence, we decided to characterize the *mabA*-encoded activity and examined, by a genetic approach, whether it can be a target for INH and ETH in *M. tuberculosis*.

METHODS

Strains, plasmids and media. Strains and plasmids used in this study are shown in Table 1. All the antibiotics used were purchased from Sigma if not mentioned otherwise. Luria

broth (Difco) and Luria agar containing 50 µg ampicillin ml⁻¹ were used for the growth of *Escherichia coli* strains (Sambrook *et al.*, 1989). Mycobacteria were grown in liquid culture using M-OADC-TW broth (Jacobs *et al.*, 1991). Middlebrook 7H10 agar (supplemented with 10% OADC and 0.5% glycerol) was used for growth on solid media, colony titrations and plating transformations of mycobacteria. Selections for mycobacteria were performed using this solid medium containing kanamycin (10–25 µg ml⁻¹), INH (0.2, 1, 10, 20 and 50 µg ml⁻¹) and ETH (10, 30, 50, 100 and 200 µg ml⁻¹). Cycloheximide was added to liquid and solid media at a final concentration of 50 µg ml⁻¹ to inhibit growth of moulds.

DNA manipulation, cloning and sequencing. The original *M. smegmatis mabA-inhA* cosmids were isolated from a *M. smegmatis* genomic library by their ability to confer INH resistance in the *M. smegmatis* host (Banerjee *et al.*, 1994). Analogous *M. tuberculosis* and *Mycobacterium bovis* BCG cosmids were obtained by screening genomic libraries of these two organisms using colony blotting. The plasmids used in this study (Table 1) are subclones of these parent cosmids. For subcloning, cosmid DNAs were digested with the restriction enzymes (New England Biolabs) indicated in Table 1, and the desired DNA fragments were isolated by using the GENECLON II kit (Bio 101) after electrophoresis on a 1% SEAKEM (FMC) agarose gel following the manufacturer's

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype/phenotype/description	Reference/source
Strains		
<i>E. coli</i> DH5α	F ⁻ <i>endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relAΔ</i> (argF-lacZYA)U169	Bethesda Research Laboratories
<i>E. coli</i> Epicurian Coli SCS1	<i>recA1 endA1 gyrA96 thi-1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 relA</i>	Stratagene
<i>E. coli</i> BL21(DE3)pLysS	A lysogenic strain containing the gene for T7 RNA polymerase under the control of a <i>lacUV</i> promoter; F ⁻ <i>ompT hsdSB</i> (r _B ⁻ m _B ⁻) <i>gal dcm</i> (DE3) pLysS (Cm ^R)	Novagen
<i>M. smegmatis</i> mc ² 6 ATCC607	Wild-type strain from which the <i>M. smegmatis mabA</i> and <i>inhA</i> genes were cloned	W. Jones, Centers for Disease Control, Atlanta, GA, USA
<i>M. smegmatis</i> mc ² 155	Electroporation-efficient mutant of mc ² 6, wild-type for drug-resistance	Snapper <i>et al.</i> (1990)
<i>M. bovis</i> BCGPasteur	Wild-type	World Health Organization, Geneva
<i>M. tuberculosis</i> H37Rv TMC102	Wild-type, causes disease in guinea pigs	Trudeau Institute
Plasmids		
pMD31	<i>E. coli</i> -mycobacteria shuttle plasmid vector with blue/white selection, ~ 7 kb, derivative of pUC119	Donnelly-Wu <i>et al.</i> (1993)
pBluescript II KS(+)	<i>E. coli</i> sequencing vector with blue/white selection	Stratagene
pET3d	<i>E. coli</i> expression vector with T7 promoter	Novagen
pET3d- <i>mabA</i>	pET3d::744 bp <i>mabA</i> PCR fragment from <i>M. tuberculosis</i> H37Rv	This work
pYUB376	pMD31::3.5 kb <i>NheI</i> insert containing <i>M. tuberculosis</i> H37Rv <i>mabA</i> and <i>inhA</i> genes	Banerjee <i>et al.</i> (1994)
pYUB630	pYUB376 derived, insert DNA 3' to <i>inhA</i> removed by digestion with <i>XbaI</i> and <i>HindIII</i> (Fig. 4)	This work
pYUB631	<i>DpnI</i> fragment carrying the CAT gene (Bartolome <i>et al.</i> , 1991) inserted into the <i>SphI</i> site of <i>mabA</i> of pYUB630 (Fig. 4)	This work
pYUB632	Derivative of pYUB376 with a truncated <i>inhA</i> gene produced by digestion with <i>BsaBI</i> and <i>HindIII</i> (Fig. 4).	This work
pYUB633	As pYUB630 except that the <i>XbaI</i> - <i>HindIII</i> fragment is from <i>M. bovis</i> BCG	This work

recommendations. Purified DNA fragments were ligated into appropriate cloning vectors (Table 1) using T4 DNA ligase (New England Biolabs) to obtain the subclones. The CaCl_2 -facilitated transformations of DNA into *E. coli* DH5 α were done following standard protocols (Sambrook *et al.*, 1989). DNA was electroporated into *M. smegmatis* following a previously published protocol (Snapper *et al.*, 1990). Chromosomal DNA for PCR experiments and Southern blots was obtained from mycobacterial cells by chemical disruption of cell walls using the protocol of van Soolingen *et al.* (1991). DNA sequencing was performed on double-stranded DNA templates using the Automated Taq DyeDeoxy Sequencing kit (Applied Biosystems) following the manufacturer's protocols. A Perkin-Elmer model 480 Thermal Cycler was used for PCR reactions and Applied Biosystems PRISM 373 sequencing apparatus was used for DNA sequencing. All DNAs were sequenced on both strands. Nucleotide sequence was compiled and analysed using the Genetics Computer Group sequence analysis software (Genetic Computer Group, 1995). *mabA* genes were translated using the TRANSLATE program to obtain the amino acid sequences of the putative MabA proteins and the peptide sequences were aligned using the Megalign program of the Lasergene software package (DNASTar).

Construction of the MabA-overproducing plasmid pET3d-mabA. The *mabA* gene product from *M. tuberculosis* was overproduced using the T7 polymerase system, as described previously (Studier *et al.*, 1990). The *mabA* gene was subcloned into the pET3d vector by PCR using *Taq* DNA polymerase (Perkin Elmer). Restriction sites were introduced at both ends of the amplified sequence to facilitate cloning. PCR was performed on plasmid pYUB376 template DNA. The N-terminal primer contained the first 24 bp of the *mabA* coding sequence and an *NcoI* restriction site overlapping the initiation codon. The C-terminal primer contained a *HindIII* restriction site, the termination codon and 20 bp of the *mabA* anti-codon sequence. The PCR reactions contained 6 ng template DNA, 1.5 μg each primer, 200 μM dNTPs, 10 μl 10 $\times$ buffer for *Taq* polymerase, 5 units *Taq* polymerase, 7 mM MgCl_2 and 10% DMSO (Perkin Elmer) in a total volume of 100 μl . The reactions were incubated at 94 °C for 1.5 min, 62 °C for 1.5 min and 72 °C for 1.5 min for 30 cycles. The products were separated on a 1% SEAKEM agarose (FMC) gel and a 744 bp PCR product was recovered using the GENECLEAN II kit (BIO 101).

The PCR product was cut with *NcoI* and *HindIII* and ligated into the previously cut and gel-purified pET3d (Table 1) at room temperature overnight. The ligation mixture was used to transform supercompetent *Escherichia coli* SCS1 cells and colonies resistant to 50 μg ampicillin ml^{-1} were selected. The plasmid was isolated from selected colonies and analysed for the presence of a 744 bp insert by 1% agarose gel electrophoresis. The resulting plasmid with the 744 bp insert was transformed into the T7 polymerase containing BL21(DE3)-pLysS host cells (Table 1).

Overexpression of MabA protein and the assay for KAR activity. Six litres of terrific broth [12 g bacto-tryptone (Difco), 24 g yeast extract (Difco), 0.4% (v/v) glycerol, 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4] containing 50 μg ampicillin ml^{-1} and 34 μg chloramphenicol ml^{-1} was inoculated with 120 ml overnight culture. The cells were grown at 37 °C to an OD_{600} of approximately 0.7. IPTG was added to a final concentration of 1 mM to induce transcription of the T7 polymerase. The cells were harvested by centrifugation at 5000 r.p.m. (Sorvall GS-3 rotor) 2.5 h after induction. Cells were resuspended in lysis buffer containing 50 mM Tris/HCl, pH 8.0, 1 mM EDTA, 0.5% Nonidet P-40 and 10 mM DTT

(Sigma). The suspension was recentrifuged at 20000 r.p.m. for 1 h in a Sorvall SS-34 rotor at 4 °C to remove the cell debris. Nucleic acids were removed from the cell-free extract by 1% streptomycin sulfate precipitation, followed by centrifugation at 20000 r.p.m. in a Sorvall SS-34 rotor for 1 h at 4 °C. The resulting supernatant was dialysed against 20 mM TAE, pH 7.8, overnight at 4 °C. The resulting soluble extracts were analysed by gel electrophoresis using 10–15% gradient polyacrylamide PhastGel (Pharmacia) along with the LMW molecular mass standard (Pharmacia).

KAR assays were performed in a quartz cuvette at 30 °C in a final volume of 1 ml with the following components: 1 mM acetoacetyl-CoA (Sigma), 40 mM HEPES, pH 7.0, and 150 μM NADH or NADPH. Normalized enzyme samples (20 μl) from induced or uninduced cellular extracts mentioned above were added to the reaction mixture. The conversion of NADH or NADPH to NAD or NADP was measured spectrophotometrically as the reduction in A_{340} by a continuous scanning method using a UVIKON 933 spectrophotometer (Kontron Instruments). The activity tests were then repeated with increasing concentrations of the induced extract using NADPH. Thereafter, initial velocities (μmol NADPH min^{-1}) of these reactions were plotted as a function of cell extract concentration. The volume of the extract used from the induced cells was 10, 15, 20 and 30 μl . The assays were repeated on the same extracts without the acetoacetyl-CoA substrate and without the NADH/NADPH cofactor as appropriate controls.

Determination of the MIC of INH and ETH to mycobacterial strains. MIC assays were performed following a modification of our previously published assay (Banerjee *et al.*, 1994). In short, mycobacterial cells bearing the indicated plasmids were grown in M-OADC-TW containing kanamycin (15 μg ml^{-1}) and dilutions were plated on 7H10 agar plates containing kanamycin (15 μg ml^{-1}) alone or kanamycin with various concentrations of INH or ETH. The strains without any plasmid were grown in M-OADC-TW broth, and dilutions were plated on 7H10 agar plates and 7H10 agar plates containing various concentrations of INH or ETH. Here, MIC is defined as the concentration of the drug that produced >99% inhibition of bacterial growth when inocula of ~ 1000 organisms were plated. Similar sized inocula were used in every assay because MICs to INH and ETH vary considerably with large variations in inoculum.

RESULTS

Cloning and characterization of *mabA* genes from *M. smegmatis*, *M. tuberculosis* and *M. bovis* BCG

The report of cloning the *inhA* gene indicated the presence of an ORF, *orf1/mabA*, upstream of *inhA* in *M. tuberculosis*, *M. bovis* and *M. smegmatis* (Banerjee *et al.*, 1994). Also, in that report, subcloning analysis demonstrated that, unlike in *M. smegmatis*, these two genes form an operon in *M. tuberculosis* and *M. bovis*. Cosmid clones were identified at that time which contained both *mabA* and *inhA* from these bacteria. In this study, parts of these cosmid inserts containing *mabA* were subcloned into pBluescript II KS(+) (Table 1) and the sequence upstream of *inhA* was determined (Fig. 1). We compared these sequences with each other, as well as with the recently released *mabA* sequence (accession no. AF002133) from *Mycobacterium avium*. The DNA region between the end of *mabA* and the

Fig. 1. DNA sequence similarity between the *mabA* genes from *M. smegmatis* mc²155, *M. tuberculosis* H37Rv and *M. bovis* BCG. The top sequence is of the *mabA* gene from *M. smegmatis* (*Msm*). The middle sequence is of the *mabA* gene from *M. tuberculosis* (*Mtb*). The *M. bovis mabA* sequence is not shown separately because it is identical to the *mabA* sequence from *M. tuberculosis*. The bottom sequence is of the *mabA* gene from *M. avium* (*Mav*). START, hypothetical translation start site; RBS, putative ribosome-binding site; STOP, hypothetical translation termination site. These sites are marked by underlines. Vertical bars between bases indicate identity; dots indicate gaps in the alignment. The base positions are indicated on the left. *M. smegmatis mabA* may also start from the GTG two codons downstream from the designated start.

beginning of *inhA* from *M. avium* is more similar to that of *M. smegmatis* than *M. tuberculosis*. This strongly indicates that, unlike the *M. tuberculosis/M. bovis mabA-inhA* operon, *mabA* and *inhA* are independently transcribed in *M. avium* as in *M. smegmatis*. This is consistent with the previous observation that the *M. avium mabA-inhA* plasmids confer a similar level of resistance to INH and ETH as *M. smegmatis mabA-inhA* plasmids, which is considerably higher than the resistance conferred by the plasmids from *M. tuberculosis* and *M. bovis* (Banerjee *et al.*, 1994). These gene organization data also correlate well with similar MICs for INH in *M. smegmatis* and *M. avium*, which are much higher than the MICs of *M. tuberculosis* complex. Moreover, like the *M. smegmatis* ORF, the *M. avium mabA* ORF was found to be longer (765 bp, 255 aa) than that of *M. tuberculosis* and *M. bovis* (741 bp, 247 aa). In *M. smegmatis*, the reading frame probably starts with an ATG, although there is a possible GTG start site present two codons downstream. The hypothetical ATG start codon gives a size conservation (255 aa) between the MabAs of *M. smegmatis* and *M. avium*. There are significant differences between the DNA sequences in the region upstream of the *mabA* ORFs as well as in the beginning of the ORFs.

Conservation of *mabA* and MabA among different mycobacteria

As in *inhA* (Banerjee *et al.*, 1994), we found that the DNA sequence of the *mabA* gene from *M. tuberculosis* is identical to that of *M. bovis* BCG (Fig. 1); we will

therefore refer to these two genes as *M. tuberculosis mabA*. Despite the difference in gene organization and translational start site, the *mabA* gene appears to be highly conserved between different mycobacteria. At the DNA level, the identity was approximately 80% (Fig. 1), which is in agreement with the high degree of homology indicated by the Southern blot using a fragment containing *mabA*. In all the 11 mycobacteria tested thus far, including important pathogens like *Mycobacterium leprae*, *M. avium* and *Mycobacterium intracellulare*, bands hybridizing with the *M. smegmatis mabA* probe were observed (data not shown). Such conservation of *mabA* among various mycobacteria is consistent with our repeated failure to produce a knockout of this gene in *M. smegmatis*. However, the chromosomal gene could easily be knocked out when an extra episomal copy was present (data not shown). These results indicate that MabA is likely to be essential, like *InhA*.

At the amino acid level (Fig. 2) the homology was even greater (82–85% identity). Expectedly, *M. tuberculosis* MabA shows stronger homology to *M. avium* MabA than the *M. smegmatis* protein, since the former two organisms share a closer ancestry. Additionally, the similarity score, indicating amino acid residue conservation between the MabAs, is about 88–90%. Apart from the first few residues of the reading frames, homology is uniform throughout the protein sequence. All the MabA peptides contain an NADP-binding consensus sequence GXGXXAXXXAXXXXXXG (Hanukoglu & Gutfinger, 1989).

1	MT--DTATENTTESAADYGRFAFVSRSVLVTGGNGIGLAIAORLAEEAH	avMab
1	MT--ATATGGA--KPFVFSRSVLVTGGNGIGLAIAORLAADGH	tbMab
1	MT--VTDFPADAGLATAGRFAFVSRSVLVTGGNGIGLAIAORLAADGH	smMab
1	MNFE-----GKIALVTGASRGIGRAIAETLAAARGG	ecFab
1	MNLE-----GKIALVTGASRGIGRAIAELLVERGA	vhFab
1	MQ-----GKIALVTGSTRGIGRAIAEELSSKGA	hiFab
49	--KVAVTHRSGAPDGLFGVEC-----DVTDDNDVDRAPTEVEEHQGPV	avMab
49	--KVAVTHRSGAPKGLFGVEC-----DVTDSDAVDRAFTAVEEHQGPV	tbMab
49	--KVAVTHRSGAPDGLFGVC-----DVTDSAGVDRAPTEVEEHQGPV	smMab
31	KVIGTATSENGAQAISDYLGANGKGLMLNVTDPASIESVLEKIRAEFGELV	ecFab
31	TVIGTATSEGGAAAISEYLGENGKGLMLNVTDVESIEATLKTINDECGAI	vhFab
29	FVIGTATSEKGAELISAYLGDGKGLVLNVTDKESIEETLEQIKNDFGDI	hiFab
91	EVLVSNAIGISKDAFLIRMTERR--TEVINANLTGAFRVQORASRSHQKKR	avMab
91	EVLVSNAIGISADAFIRMTERR--TEVINANLTGAFRVQORASRSMQRNK	tbMab
91	EVLVSNAIGISKDAFLIRMTERR--TEVINANLTGAFRVQORASRSMQRNK	smMab
81	DILVNNAIGITRDNLTMRMKDDEWN-DIETNLTSSVFRLSKAVMRAHMKKR	ecFab
81	DILVNNAIGITRDNLTMRMKDDEWN-DIETNLTSSVFRLSKAVMRAHMKKR	vhFab
79	DILVNNAIGITRDNLTMRMKDDEWN-DIETNLTSSVFRLSKAVMRAHMKKR	hiFab
140	FGRITVYGSVSGMWITGNQANYAAAKAGLIGMARSIARELSKAGVTANV	avMab
132	FGRMIFVGSVSGMWITGNQANYAAAKAGLIGMARSIARELSKAGVTANV	tbMab
140	FGRITVYGSVSGMWITGNQANYAAAKAGLIGMARSIARELSKAGVTANV	smMab
130	HGRITVYGSVSGMWITGNQANYAAAKAGLIGFSSKSLARVVASRGITVNV	ecFab
130	HGRITVYGSVSGMWITGNQANYAAAKAGLIGFSSKSLARVVASRGITVNV	vhFab
128	FGRITVYGSVSGMWITGNQANYAAAKAGLIGFSSKSLARVVASRGITVNV	hiFab
190	APGVYIDTEMTALDERITQAGALEFTPAKRVTGAEEVPGAVSFLASEDASY	avMab
182	APGVYIDTEMTALDERITQAGALEFTPAKRVTGAEEVPGAVSFLASEDASY	tbMab
190	APGVYIDTEMTALDERITQAGALEFTPAKRVTGAEEVPGAVSFLASEDASY	smMab
180	APGVYIDTEMTALDERITQAGALEFTPAKRVTGAEEVPGAVSFLASEDASY	ecFab
180	APGVYIDTEMTALDERITQAGALEFTPAKRVTGAEEVPGAVSFLASEDASY	vhFab
178	APGVYIDTEMTALDERITQAGALEFTPAKRVTGAEEVPGAVSFLASEDASY	hiFab
240	TAGAVIPVDGGMGMGH	avMab
232	ISGAVIPVDGGMGMGH	tbMab
240	TAGAVIPVDGGMGMGH	smMab
230	ITGTEFLHVNNGGMVHV	ecFab
230	ITGTEFLHVNNGGMVHV	vhFab
228	ITGTEFLHVNNGGMVHV	hiFab

Fig. 2. Comparison of amino acid sequences of the mycobacterial MabA polypeptide with the KAR or *fabG* gene-product of *E. coli*, *H. influenzae* and *V. harveyi*. From top to bottom, the sequences presented are MabAs from *M. avium* (avMab), *M. tuberculosis* (tbMab) and *M. smegmatis* (smMab), and KARs from *E. coli* (ecFab), *V. harveyi* (vhFab) and *H. influenzae* (hiFab). The comparison was performed using the Jotun-Hein algorithm of the Megalign program of Lasergene software package from DNAstar. The boxes indicate identical amino acids. The putative NADPH-binding GXG-XXAXXXAXXXXXXG consensus sequence (Hanukoglu & Gutfinger, 1989) is indicated by a dashed overline.

Homology of MabA amino acid sequence with other KARs that are available in the database

A TFasta (Genetics Computer Group, 1995) search was performed on the EMBL and GenBank databases, using both the amino acid sequences. The search revealed that the best homologies to MabA are KARs from *E. coli* (Rawlings & Cronan, 1992), *Vibrio harveyi* (Shen & Byers, 1996) and *Haemophilus influenzae* (Fleischmann *et al.*, 1995). The sequence alignment of these three fatty acid biosynthesis enzymes with the three MabA sequences is shown in Fig. 2. The identity between the MabAs and the KARs is approximately 40% and the total similarity is about 60%. In addition, the KAR counterparts from plants, the β -ketoacyl-ACP reductases of *Cuphea lanceolata* (Klein *et al.*, 1992), *Arabidopsis thaliana* and *Brassica napus* (Slabas *et al.*, 1992), show strong homology (~35–40% identity) to the two MabA sequences.

Overexpression of MabA and biochemical determination of its activity

The 744 bp insert in the pET3d-*mabA* construct was verified by sequencing and found to match the wild-type *mabA* sequence. To verify the function of the reading frame in the construct, pET3d-*mabA* was transformed into BL21(DE3)pLysS, a lysogenic strain containing the gene for T7 RNA polymerase under the control of a lacUV5 promoter. Cell-free extracts made from cultures after 2.5 h with or without induction by 1 mM IPTG were examined by PAGE. The IPTG-induced cells harbouring pET3d-*mabA* showed moderate overexpression of a 28 kDa protein (data not shown) which matched the predicted molecular mass of 25665 Da. This protein band was not seen in extracts of uninduced cells or control cells without plasmids.

Fig. 3. Characterization of the enzymic activity of MabA protein from *M. tuberculosis*. Continuous scan assay of relative KAR activity using NADH or NADPH as cofactor in the presence or absence of IPTG induction. A_{340} is plotted as a function of reaction time for (1) induced culture with NADPH but without acetoacetyl-CoA, (2) uninduced cell extract + NADH, (3) uninduced cell extract + NADPH, (4) induced cell extract + NADH and (5) induced cell extract + NADPH.

The soluble extracts from both induced and uninduced strains were assayed for KAR activity. The extracts were tested in the presence of either NADH or NADPH. By a continuous scanning spectrophotometric assay, using both cofactors, the extracts from the induced cells were found to have considerable KAR activity (Fig. 3). However, the enzyme demonstrated a definite preference for NADPH, as seen by the sevenfold increase in activity in the presence of this substrate. This definite preference for NADPH agrees with the presence of an NADP-binding motif (Hanukoglu & Gutfinger, 1989) in MabA (Fig. 2). The extract from the uninduced cells showed no significant activity although there could be some back-

Fig. 4. Subcloning analysis to determine whether *mabA* from *M. tuberculosis* can confer resistance to INH and ETH. The top line indicates the *inhA-mabA* locus. The solid arrows indicate the ORFs and the thin line indicates noncoding regions. The open bars represent different restriction fragments of *M. tuberculosis* DNA that were cloned into the vector pMD31. B, *Bam*HI; Bs, *Bsa*BI; N, *Nhe*I; S, *Sph*I; X, *Xba*I. The orientation of the CAT gene in pYUB631 is indicated by an arrow.

ground activity from the *E. coli* KAR. The host activity is probably not observed because the *E. coli* KAR, which preferentially uses acetoacetyl-ACP, has been reported to have very poor activity with acetoacetyl-CoA as substrate (Toomey & Wakil, 1966). In addition, induction of this activity by IPTG clearly distinguished the KAR of *M. tuberculosis* from any background contaminant from the *E. coli* host or other contaminating activities in the extract. The activity tests were then repeated with increasing concentrations of the induced extract and demonstrated a linear dependence (data not shown) of activity on the amount of the induced extract (i.e. MabA protein concentration) to further support the claim that the observed KAR activity is indeed from the induced MabA protein.

Examination of the multiple copy effect of *M. tuberculosis mabA* on INH-ETH resistance

New plasmids were constructed based on the mycobacteria-*E. coli* shuttle vector pMD31 (Donnelly-Wu *et al.*, 1993) to separate *mabA* from *inhA* (Fig. 4). The first of these new plasmids, pYUB630, is a derivative of a previously published plasmid, pYUB376 (Table 1). It contains the entire *mabA-inhA* locus but none of the extra DNA present 3' of *inhA* in pYUB376 and it confers the INH-ETH-resistance phenotype in the *M. smegmatis* mc²155 host by raising the MIC_{INH} to 15 µg ml⁻¹ and MIC_{ETH} to 30 µg ml⁻¹ from the wild-type values of 5 µg ml⁻¹ and 10 µg ml⁻¹ (Fig. 4). A chloramphenicol acetyltransferase (CAT) cassette (Bartolome *et al.*, 1991) was inserted into *mabA* in pYUB630. The CAT fragment is known to be nonpolar and does not carry any terminator of transcription. Hence, this insertion should not interfere with the expression of *inhA*. This construct with a defective *mabA* but a wild-type *inhA* was designated pYUB631. When introduced into mc²155, pYUB631 conferred the same resistance phenotype as

pYUB630. pYUB632 contains a wild-type *mabA* and a truncated *inhA*. This plasmid failed to confer any drug resistance upon the host. The cloning vector pMD31 by itself did not confer any resistance to INH and ETH when introduced into mc²155. In addition, pYUB632 was tested in *M. bovis* BCG, where it also failed to show any enhanced resistance over the pMD31 vector control (MIC_{INH} 0.2 µg ml⁻¹).

The results discussed above clearly show that interruption of *M. tuberculosis mabA* without affecting *inhA* does not interfere with the ability of the plasmid to confer INH-ETH resistance. However, a deletion of the *inhA* locus while keeping *mabA* intact causes a complete loss of the resistance phenotype. Therefore, unlike the overexpression of *M. tuberculosis* *InhA*, the overexpression of MabA from *M. tuberculosis* does not show the ability to titrate INH and ETH.

DISCUSSION

In *M. tuberculosis* and *M. bovis*, *mabA* and *inhA* are in one operon whereas in *M. smegmatis* (and probably in *M. avium*) these two genes are independently transcribed (Banerjee *et al.*, 1994). As discussed in the Introduction, MabA from *M. tuberculosis* was suggested as a possible target for INH, mainly due to frequent observations of mutations at the RBS of *mabA* in many clinical INH-resistant *M. tuberculosis* isolates (Musser, 1995). This hypothesis was further supported by the unique *mabA-inhA* operon arrangement in the INH-sensitive *M. tuberculosis* complex, the similarity in the degree of MabA conservation with *InhA* conservation among different mycobacteria and the probable essential nature of MabA activity. Additionally, this possibility was supported by a biochemical study on *M. avium* (Kikuchi *et al.*, 1989) which reported that INH inhibits a KAR in addition to an ER. This KAR, like

MabA, was demonstrated to prefer NADPH over NADH and was reported to have specific involvement in mycolic acid synthesis. Although a direct involvement of MabA in mycolate synthesis could not be tested due to our inability to procure or synthesize a long-chain substrate for the KAR assay, *mabA* probably synthesizes mycolic acid because it forms an operon with *inhA*, which has been shown to be involved in mycolate synthesis (Quemard *et al.*, 1995; Wheeler & Anderson, 1996). Finally, in *M. tuberculosis*, there is room for the presence of other INH target(s) besides *InhA* because the MIC for the *M. tuberculosis* complex is considerably lower (25- to 100-fold) than that of *M. smegmatis* (Banerjee *et al.*, 1994; Zhang *et al.*, 1992) even though *InhA*s from these organisms are highly conserved. Hence, it became imperative to test the role of MabA in INH resistance.

It has recently been clearly demonstrated that INH reacts with NADH, the preferred cofactor for *InhA*, to form isonicotinic acyl-NADH inside the active site of *InhA* (Rozwarski *et al.*, 1998). This reaction, catalysed by KatG and Mn²⁺, blocks the ER activity of the protein. ETH is proposed to bind *InhA* by a similar, but different, mechanism. These observations support the earlier hypothesis (Banerjee *et al.*, 1994) that overexpression of *InhA* can titrate additional active INH (i.e. isonicotinic acyl-NADH) and thereby increase the MIC or the level of resistance. However, data discussed in this manuscript show that overexpression of MabA from *M. tuberculosis* does not confer the ability to titrate either INH or ETH. This rules out the possibility of MabA being a direct target *in vivo* for an active INH like isonicotinic acyl-NADH/NADPH. This conclusion is consistent with the fact that, until now, no mutations have been mapped to the MabA ORF in INH-resistant organisms. In contrast, mutations have been mapped to the *mabA* RBS with high frequency and also in the *InhA* ORF with lower, but significant, frequency (Banerjee *et al.*, 1994; Ristow *et al.*, 1995; Kapur *et al.*, 1996; Rozwarski *et al.*, 1998). Additionally, a report using single-copy integrating vectors demonstrated that the mutations in the *mabA* RBS actually led to overexpression of *InhA* (Mdluli *et al.*, 1996). However, the authors concluded from their drug sensitivity assays that these mutations play no role in INH resistance. Nonetheless, on close scrutiny of their data, we see that these mutations conferred a clear increase in INH resistance, particularly at the level corresponding to the MIC of *M. tuberculosis*. Hence, we propose that mutations in the RBS of *mabA* mediate INH resistance in *M. tuberculosis* by overexpression of the *InhA* protein through transcriptional upregulation of the whole *inhA* operon and not by specific translational upregulation of MabA. This hypothesis is consistent with the fact that hundreds of INH-resistant strains from different parts of the world demonstrated *mabA* RBS mutations but none of many INH-sensitive strains screened have demonstrated these mutations (Musser *et al.*, 1996).

In addition, the observation of INH-induced inhibition of both KAR and ER in the *in vitro* system of Kikuchi *et*

al. (1989) is not necessarily contradictory to our hypothesis of MabA not being a direct and primary target of INH *in vivo*. These two systems are very different. In the *in vitro* system, KAR and ER were assayed for INH inhibition in semi-defined extracts. It is completely unknown whether those fractions contained some, or any, KatG and Mn²⁺. Moreover, the concentration of INH is much higher in the *in vitro* assay. All these important and many more subtle disagreements between the *in vitro* and the *in vivo* systems may make the mechanism of action of the *in vitro* system different from the *in vivo* model. Hence, given the complex nature of the INH mode of action, an undefined *in vitro* assay may not represent the actual *in vivo* environment.

Finally, cloning and characterization of MabA may also help in the future development of antimycobacterial therapeutics. Characterization of *InhA* activity and knowledge of its three-dimensional structure have generated a structure-function-based approach to design new antimycobacterial compounds (Rozwarski *et al.*, 1998) and broad-spectrum antibiotics (Baldock *et al.*, 1996). Similarly, the knowledge of the MabA activity together with the ability to overexpress this highly conserved protein presents the *mabA*-encoded KAR as a potential target for developing new drugs. Additionally, further biochemical and structural characterization of this KAR may also lead to greater insight into the as yet unknown but important biology of mycolic acid biosynthesis.

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REFERENCES

- Baldock, C., Rafferty, J. B., Sedelnikova, S. E., Baker, P. J., Stuitje, A. R., Slabas, A. R., Hawkes, T. R. & Rice, D. W. (1996). A mechanism of drug action revealed by structural studies of enoyl reductase. *Science* **274**, 2107–2110.
- Banerjee, A., Dubnau, E., Quemard, A., Balasubramanian, V., Um, K. S., Wilson, T., Collins, D., de Lisle, G. & Jacobs, W. R., Jr (1994). *inhA*, a gene encoding a target for isoniazid and ethionamide in *Mycobacterium tuberculosis*. *Science* **263**, 227–230.
- Bartolome, B., Jubete, Y., Martinez, E. & De La Cruz, F. (1991). Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. *Gene* **102**, 75–78.
- Dessen, A., Quemard, A., Blanchard, J. S., Jacobs, W. R., Jr & Sacchettini, J. C. (1995). Crystal structure and function of the isoniazid target of *Mycobacterium tuberculosis*. *Science* **267**, 1638–1641.
- Donnelly-Wu, M. K., Jacobs, W. R., Jr & Hatfull, G. F. (1993). Superinfection immunity of mycobacteriophage L5: applications for genetic transformation of mycobacteria. *Mol Microbiol* **7**, 407–417.
- Fleischmann, R. D., Adams, M. D., White, O. & 37 other authors (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.

- Genetics Computer Group (1995).** *Program Manual: Wisconsin Sequence Analysis Package, Version 8, UNIX*. Madison, WI: Genetics Computer Group, Madison.
- Hanukoglu, I. & Gutfinger, T. (1989).** cDNA sequence of adrenodoxin reductase. Identification of NADP-binding sites in oxidoreductases. *Eur J Biochem* **180**, 479–484.
- Heym, B., Honore, N., Truffot-Pernot, C., Banerjee, A., Schurra, C., Jacobs, W. R., Jr, van Embden, J. D., Grosset, J. H. & Cole, S. T. (1994).** Implications of multidrug resistance for the future of chemotherapy of tuberculosis: a molecular study. *Lancet* **344**, 293–298.
- Jacobs, W. R., Jr, Kalpana, G. V., Cirillo, J. D., Pascopella, L., Snapper, S. B., Udani, R., Jones, W., Barletta, R. G. & Bloom, B. R. (1991).** Genetic systems for mycobacteria. *Methods Enzymol* **204**, 537–555.
- Johnsson, K., King, D. S. & Schultz, P. G. (1995).** Studies on the mechanism of action of isoniazid and ethionamide in the chemotherapy of tuberculosis. *J Amer Chem Soc* **117**, 5009–5010.
- Kapur, V., Li, L. L., Hamrick, M. R. & 11 other authors (1996).** Rapid mycobacterium species assignment and unambiguous mutations associated with antimicrobial resistance in *Mycobacterium tuberculosis*. *Arch Pathol Lab Med* **119**, 131–138.
- Kikuchi, S., Takeuchi, T., Yasui, M., Kusaka, T. & Kolattukudy, P. E. (1989).** A very long-chain fatty acid elongation system in *Mycobacterium avium* and a possible mode of action of isoniazid on the system. *Agric Biol Chem* **53**, 1689–1698.
- Klein, B., Pawlowski, K., Horicke-Grandpierre, C., Schell, J. & Topfer, R. (1992).** Isolation and characterization of a cDNA from *Cuphea lanceolata* encoding a β -ketoacyl-ACP reductase. *Mol Gen Genet* **233**, 122–128.
- Mdluli, K., Sherman, D. R., Hickey, M. J., Kreiswirth, B. N., Morris, S., Stover, C. K. & Barry, C. E. (1996).** Biochemical and genetic data suggest that InhA is not the primary target for activated isoniazid in *Mycobacterium tuberculosis*. *J Infect Dis* **174**, 1085–1090.
- Musser, J. M. (1995).** Antimicrobial agent resistance in mycobacteria – molecular genetic insights. *Clin Microbiol Rev* **8**, 496–514.
- Musser, J. M., Kapur, V., Williams, D. L., Kreiswirth, B. N. & Vansoolingen, D. (1996).** Characterization of the catalase-peroxidase gene (*katG*) and *inhA* locus in isoniazid-resistant and -susceptible strains of *Mycobacterium tuberculosis* by automated DNA sequencing – restricted array of mutations associated with drug resistance. *J Infect Dis* **173**, 196–202.
- Quemard, A., Sacchettini, J. C., Dessen, A., Vilcheze, C., Bittman, R., Jacobs, W. R., Jr & Blanchard, J. S. (1995).** Enzymatic characterization of the target for isoniazid in *Mycobacterium tuberculosis*. *Biochemistry* **34**, 8235–8241.
- Rawlings, M. & Cronan, J. E., Jr (1992).** The gene encoding *Escherichia coli* acyl carrier protein lies within a cluster of fatty acid biosynthetic genes. *J Biol Chem* **267**, 5751–5754.
- Ristow, M., Mohlig, M., Rifai, M., Schatz, H., Feldmann, K. & Pfeiffer, A. (1995).** New isoniazid/ethionamide resistance gene mutation and screening for multidrug-resistant *Mycobacterium tuberculosis* strains. *Lancet* **346**, 502–503.
- Rouse, D. A., Li, Z. M., Bai, G. H. & Morris, S. L. (1995).** Characterization of the *katG* and *inhA* genes of isoniazid-resistant clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* **39**, 2472–2477.
- Rozwarski, D. A., Grant, G. A., Barton, D. H. R., Jacobs, W. R., Jr & Sacchettini, J. C. (1998).** Modification of the NADH of the isoniazid target (InhA) from *Mycobacterium tuberculosis*. *Science* **278**, 98–102.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shen, Z. W. & Byers, D. M. (1996).** Isolation of *Vibrio harveyi* acyl carrier protein and the *fabG*, *acpP*, and *fabF* genes involved in fatty acid biosynthesis. *J Bacteriol* **178**, 571–573.
- Slabas, A. R., Chase, D., Nishida, I. & 7 other authors (1992).** Molecular cloning of higher-plant 3-oxoacyl-(acyl carrier protein) reductase. Sequence identities with the *nodG*-gene product of the nitrogen-fixing soil bacterium *Rhizobium meliloti*. *Biochem J* **283**, 321–326.
- Snapper, S. B., Melton, R. E., Mustafa, S., Kieser, T. & Jacobs, W. R., Jr (1990).** Isolation and characterization of efficient plasmid transformation mutants of *Mycobacterium smegmatis*. *Mol Microbiol* **4**, 1911–1919.
- Snider, D. E., Raviglione, M. & Kochi, A. (1994).** Global burden of tuberculosis. In *Tuberculosis: Pathogenesis, Protection, and Control*, pp. 3–12. Edited by B. R. Bloom. Washington, DC: American Society for Microbiology.
- van Soolingen, D., Hermans, P. W., de Haas, P. E., Soll, D. R. & van Embden, J. D. (1991).** Occurrence and stability of insertion sequences in *Mycobacterium tuberculosis* complex strains: evaluation of an insertion sequence-dependent DNA polymorphism as a tool in the epidemiology of tuberculosis. *Clin Microbiol* **29**, 2578–2586.
- Studier, F. W., Rosenberg, A. H., Dunn, J. J. & Dubendorff, J. W. (1990).** Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* **185**, 60–89.
- Telenti, A., Honore, N., Bernasconi, C., March, J., Ortega, A., Heym, B., Takiff, H. E. & Cole, S. T. (1997).** Genotypic assessment of isoniazid and rifampin resistance in *Mycobacterium tuberculosis*: a blind study at reference laboratory level. *J Clin Microbiol* **35**, 719–723.
- Toomey, R. E. & Wakil, S. J. (1966).** Studies on the mechanism of fatty acid synthesis. XV. Preparation and general properties of β -ketoacyl acyl carrier protein reductase from *Escherichia coli*. *Biochim Biophys Acta* **116**, 189–197.
- Victor, T. C., Warren, R., Butt, J. L. & 9 other authors (1997).** Genome and MIC stability in *Mycobacterium tuberculosis* and indications for continuation of use of isoniazid in multidrug-resistant tuberculosis. *J Med Microbiol* **46**, 847–857.
- Wheeler, P. R. & Anderson, P. M. (1996).** Determination of the primary target for isoniazid in mycobacterial mycolic acid biosynthesis with *Mycobacterium aurum* A+. *Biochem J* **318**, 451–457.
- World Health Organization (1997).** *The World Health Report 1997*. Geneva, Switzerland: World Health Organization.
- Zhang, Y., Heym, B., Allen, B., Young, D. & Cole, S. (1992).** The catalase-peroxidase gene and isoniazid resistance of *Mycobacterium tuberculosis*. *Nature* **358**, 591–593.

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