Inactivation of *mshB*, a key gene in the mycothiol biosynthesis pathway in *Mycobacterium smegmatis*

Mamta Rawat,[1] Svetozar Kovacevic,[2] Helen Billman-Jacobe[3] and Yossef Av-Gay[1]

Correspondence
Yossef Av-Gay
yossi@interchange.ubc.ca

[1]Department of Medicine, Division of Infectious Diseases, University of British Columbia, Vancouver, British Columbia, Canada V5Z 3J5

[2]Department of Microbiology, Monash University, Clayton, Victoria 3800, Australia

[3]Department of Microbiology and Immunology, University of Melbourne, Parkville, Victoria 3010, Australia

The *mshB* gene encoding *N*-acetyl-1-D-*myo*-inosityl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase (MshB) is a key enzyme in mycothiol biosynthesis. Disruption of *mshB* in *Mycobacterium smegmatis* resulted in decreased production of mycothiol (5–10 % of the parent strain mc^{2}155) but did not abolish mycothiol synthesis completely. Complementation of the MshB$^-$ mutants with the *mshB* gene resulted in increased mycothiol production towards the exponential and stationary phases of the bacterial growth cycle. These results suggest that another enzyme is capable of mycothiol biosynthesis by providing *N*-acetylglucosaminylinositol deacetylation activity in the absence of MshB. One of the candidate enzymes capable of carrying out such reactions is the MshB orthologue mycothiol amide hydrolase, MCA. However, epichromosomal expression of *mca* in the MshB$^-$ mutants did not restore mycothiol levels to the level of the parent strain. Unlike other mutants, which have little or no detectable levels of mycothiol, the MshB$^-$ mutant did not exhibit increased resistance to isoniazid. However, the MshB$^-$ mutant was resistant to ethionamide. Phenotypic analysis of other mutants lacking mycothiol revealed that MshA$^-$ mutants also exhibit ethionamide resistance but that a MshC$^-$mutant was sensitive to ethionamide, suggesting that mycothiol or its early intermediates influence ethionamide activation.

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INTRODUCTION

In eukaryotes and Gram-negative prokaryotes, glutathione, a low-molecular-mass thiol, plays an important role in cysteine storage and defence against oxidative stress and environmental toxins. Gram-positive actinomycetes like mycobacteria do not produce glutathione but instead synthesize a low-molecular-mass thiol, mycothiol. Mycothiol consists of *N*-acetyl-L-cysteine linked to a pseudodisaccharide, D-glucosamine and *myo*-inositol (Newton *et al.*, 1996), and appears to serve similar functions to glutathione. We have previously shown that *Mycobacterium smegmatis* mutants lacking mycothiol are more sensitive to oxidative stress and antibiotic stress (Newton *et al.*, 1999; Rawat *et al.*, 2002). In addition, we have reported that mycobacteria possess a mycothiol-dependent detoxification system (Newton *et. al.*, 2000a). Mycothiol and its dependent detoxification enzymes may assist pathogenic mycobacteria, such as *Mycobacterium tuberculosis*, to survive the normally bacteriocidal reactive oxygen intermediates produced by the host's macrophages. Thus, enzymes involved in mycothiol biosynthesis are considered to be attractive drug targets (Young & Duncan, 1995).

Mycothiol biosynthesis is proposed to proceed through a four-step pathway (Bornemann *et al.*, 1997; Anderberg *et al.*, 1998; Newton & Fahey, 2002). First the formation GlcNAc-Ins is catalysed by *N*-acetylglucosamine transferase (MshA), followed by deacetylation by MshB deacetylase. The resulting GlcN-Ins is ligated with a cysteine in a reaction catalysed by a ligase, MshC (Sareen *et al.*, 2002). The Cys-GlcN-Ins is then acetylated to form mycothiol in a reaction catalysed by MshD acetyltransferase (Koledin *et al.*, 2002). Recently, the enzymes MshB from *M. tuberculosis* (mtMshB), MshC from *M. smegmatis* (msMshC) and MshD from *M. smegmatis* (msMshD) have been characterized and *M. smegmatis* mutants in *mshA* (Newton *et. al.*, 2003), *mshC*

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; DIG, digoxigenin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); GlcNAc-Ins, 1-D-*myo*-inosityl-2-acetamido-2-deoxy-α-D-glucopyranoside; GlcN-Ins, 1-D-*myo*-inosityl-2-deoxy-α-D-glucopyranoside; LB, Lennox L broth; mBBr, monobromobimane; MCA, mycothiol amide hydrolase; OADC, oleic acid, albumin, dextrose [glucose], catalase supplement; TSB, trypticase soy broth.

(Rawat *et al.*, 2002) and *mshD* (Koledin *et al.*, 2002) have been isolated. The *mshB* gene was identified based upon its homology to the mycothiol-dependent amidase MCA, which is encoded by the *M. tuberculosis* ORF Rv1082 (Cole *et al.*, 1998). *M. tuberculosis* MshB was cloned and expressed in *Escherichia coli* and the recombinant mtMshB was shown to possess GlcNAc-Ins deacetylase activity *in vitro* (Newton *et al.*, 2000b). Although mtMshB was the first enzyme to be identified and characterized we were not able to identify *mshB*-defective mutants by screening transposon and chemical mutant libraries of *M. smegmatis*. To test whether it is possible to generate such mutants, and to determine the role of MshB within living mycobacterial cells, we constructed an *mshB*-deficient strain of *M. smegmatis*. In this study we describe the creation and characterization of the *mshB* mutant.

METHODS

Bacterial strains and culture conditions. *E. coli* strains DH5α, JM109 and InvαF′ were used as host strains for cloning experiments. *E. coli* strains were grown on LB medium. *M. smegmatis* mc^{2}155 (Snapper *et al.*, 1988) was grown in Middlebrook 7H9 broth (Difco) with 0·05 % Tween, Middlebrook 7H10 agar or trypticase soy broth (TSB) with 0·5 % Tween. Middlebrook medium was supplemented with OADC supplement (Difco) or 1 % (w/v) glucose. Ampicillin (100 μg ml^{-1}), gentamicin (15 μg ml^{-1}), kanamycin (20–25 μg ml^{-1}), streptomycin (20 μg ml^{-1}) and hygromycin (100 μg ml^{-1} for *E. coli* and 50 μg ml^{-1} for *M. smegmatis*) were added as required. For induction of expression in cell strains of *M. smegmatis* with genes cloned in the pALACE vector (De Smet *et al.*, 1999), 1 % (w/v) acetamide was added to Middlebrook 7H9 broth with 0·05 % Tween.

Molecular biology techniques. Strains, plasmids and oligonucleotides used in this study are described in Table 1. Genomic DNA was isolated from *M. smegmatis* cultures according to Larsen (2000) and as previously described (Billman-Jacobe *et al.*, 1999). *M. smegmatis* transformations were carried out with a Bio-Rad Gene Pulser using mycobacterial cells prepared as described by Snapper *et al.* (1988). Standard recombinant DNA techniques such as restriction digests, ligations and transformations were carried out as described by Sambrook *et al.* (1989). Probes for Southern blotting were labelled with digoxigenin (DIG)-labelled dNTPs using a Roche DIG labelling kit, and membranes developed according to the manufacturer's instructions.

Disruption of the *mshB* gene in *M. smegmatis*. A recombination cassette was constructed in order to disrupt *mshB* on the *M. smegmatis* chromosome. A 2·5 kbp *Dde*I DNA fragment was subcloned from an exisiting plasmid obtained from a partial genomic library (data not shown). The *Dde*I fragment spanned from 773 bp upstream to 906 bp downstream of the *mshB* ORF. The cohesive ends of the fragment were filled with T4 DNA polymerase and cloned into *Kpn*I-digested, T4 DNA polymerase-treated pUC18. The resulting plasmid, pHBJ273, was then linearized by digestion at a unique *Kpn*I site within the *mshB* ORF and the ends were filled as above. A kanamycin-resistance cassette was prepared by PCR amplification of the *aph* gene from pUC18K using M13 universal and reverse primers and then the 850 bp PCR product was digested with *Kpn*I and *Hin*cII and the *Kpn*I cohesive end was filled as above. The kanamycin-resistance cassette was ligated with pHBJ273 such that the resulting plasmid, pHBJ278, had the cassette disrupting the *mshB* ORF in a non-polar fashion to avoid affecting the transcription of downstream genes. A streptomycin-resistance marker (Prentki & Kirsch, 1984) was cloned into a *Hin*dIII site in the vector portion of pHBJ278 to provide a selectable marker in mycobacteria, resulting in the final plasmid pHBJ280.

Toxicity and antibiotic sensitivity studies. The minimal inhibitory concentrations (MICs) of ethionamide and isoniazid (Sigma) were determined for the *M. smegmatis* wild-type (WT) and derivatives. Exponentially growing cultures were serially diluted and applied to Middlebrook 7H10 plates containing various concentrations of antibiotics using a replicator. An inoculum of 500–1000 c.f.u. per spot was used and the MICs were read as the lowest concentration that inhibited at least 98·8 % of growth. All MICs were tested in duplicate at least twice.

Disk diffusion assays, as described by Rawat *et al.* (2002), were also used to determine the sensitivity of *M. smegmatis* wild-type and mutants to ethionamide, cerulenin and vancomycin and to various oxidative and alkylating stress generators: diamide (1·0–10·0 μmol), cumene hydroperoxide (0·1–5·0 μmol), menadione (0·01–1·0 μmol), nitrofurantoin (0·1–10·0 μmol), plumbagin (0·01–1 μmol), DTNB (0·01–1·0 μmol), CDNB (0·01–1·0 μmol), mBBr (0·5–5·0 μmol) and iodoacetamide (0·01–0·1 μmol). The disk diffusion assays were performed in triplicate at least three times.

Determination of mycothiol levels. Derivatization of cell extracts with mBBr to determine the thiol content was performed essentially as described by Anderberg *et al.* (1998). Briefly, cell pellets were suspended in 50 % aqueous acetonitrile, 2 mM mBBr, 20 mM HEPES (pH 8·0). The suspensions were then incubated in the dark at 60 °C for 15 min and after the incubation period, 2 μl concentrated 12 M HCl was added to acidify the suspensions. The suspensions were then centrifuged to collect the cell debris and the supernatant was diluted with 10 mM HCl and subjected to HPLC analysis. Control samples were extracted with 50 % aqueous acetonitrile, 5 mM *N*-ethylmaleimide and 20 mM HEPES (pH 8·0). The suspensions were incubated for 15 min at 60 °C. After addition of 2 mM mBBr, the suspensions were incubated again for 15 min at 60 °C. The suspensions were then centrifuged to collect the cell debris and the supernatant was diluted with 10 mM HCl and subjected to HPLC analysis on a Beckman Ultrasphere C18 ion-pair HPLC column. The thiols were eluted with 0·25 % glacial acetic acid pH 3·6 (buffer A) and 100 % methanol (buffer B) using the following gradient: initial conditions, 10 % buffer B; at 15 min, 18 % buffer B; at 30 min, 27 % buffer B; at 33 min, 100 % buffer B; at 36 min, 10 % buffer B; and at 52 min, 10 % buffer B and reinjection. The flow rate was 1 ml min^{-1} and fluorescence detection was as described previously (Anderberg *et al.*, 1998).

Complementation of Myco504 with *M. smegmatis mshB* homologue. The *M. smegmatis mshB* ORF was PCR amplified from *M. smegmatis* mc^{2}155 genomic DNA using primers 1 and 2 (Table 1). The PCR product included 300 bp upstream of the ORF and was presumed to contain the *mshB* promoter. Sites were included in the primers to facilitate cloning into pGINT, an integrative *M. smegmatis–E. coli* shuttle vector derived from the pHINT vector (O'Gaora *et al.*, 1997). The *M. smegmatis mshB* gene was PCR amplified and the PCR product was cloned into pCR2.1. The *mshB* gene was then excised using *Hin*dIII and *Xho*I and ligated with *Hin*dIII- and *Xho*I-digested pGINT to create pM3. Myco504, the MshB$^-$ mutant, was transformed with pM3 and kanamycin/gentamicin-resistant transformants were selected. The mycothiol content of the transformants was measured and one transformant, named Myco504*mshB*, was selected for further study. We cannot eliminate the possiblity that some transcriptional readthrough from a vector-encoded promoter could occur and regarded the expression as uncontrolled expression.

Expression of *M. tuberculosis mca* in Myco504. The *M. tuberculosis mca* gene was PCR amplified using primers 3 and 4 (Table 1)

Table 1. Strains, plasmids and oligonucleotides used in this study

	Characteristics	Source or reference
Strains		
E. coli		
DH5α	F$^-$ *gyrA96* (Nal^r) *recA1 relA1 endA1 thi-1 hsdR17* (r_{kk}^- m_k^+) *glnV44 deoR* Δ(*lacZYA–argF*)*U169* [ϕ80d Δ(*lacZ*)M15]	
JM109	F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ(*lacZ*) M15/ e14$^-$ (McrA$^-$) Δ(*lac–proAB*) *endA1 gyrA96* (Nal^r) *thi-1 hsdR17* (r_k^- m_k^+) *glnV44 recA1 relA1*	
InvαF′	F′ *endA1 recA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 gyrA96 relA1* ϕ80*lacZ*ΔM15 Δ(*lacZYA–argF*)U169λ	
M. smegmatis		
mc^{2}155	Wild-type parent	Snapper *et al.* (1988)
Myco504	*mshB*::*aph*	This study
Myco504*mshB*	Myco504/pM3	This study
Myco504*mca*	Myco504/pM4	This study
WT*mca*	mc^{2}155/pM4	This study
A1	*mshA*	Newton *et al.* (2003)
A1c	*mshA* complemented with *mshA*	Newton *et al.* (2003)
49	*mshA*	Newton *et al.* (2003)
49c	*mshA* complemented with *mshA*	Newton *et al.* (2003)
I64	*mshC*	Rawat *et al.* (2002)
Vectors		
pALACE	*E. coli–Mycobacterium* shuttle vector, Hyg^r	De Smet *et al.* (1999)
PGINT	*E. coli–Mycobacterium* shuttle vector, Gent^r	O'Gaora *et al.* (1997)
pCR2.1	Cloning vector, Amp^r Kan^r	Invitrogen
pUC18	Cloning vector, Amp^r	
Plasmids		
pHBJ273	2·5 kb *Dde*I fragment containing *M. smegmatis mshB* cloned into pUC18	This study
pHBJ278	PHBJ273 with *aph* inserted in *mshB*	This study
pHBJ280	PHBJ278 with *str* cloned into a *Hin*dIII site	This study
pM1	*M. tuberculosis mca* cloned into pCR2.1	This study
pM2	*M. smegmatis mshB* cloned into pCR2.1	This study
pM3	*M. smegmatis mshB* cloned into pGINT	This study
pM4	*M. tuberculosis mca* cloned into pALACE	This study
Oligonucleotides		
1	5′-TTAAGCTTCGCCGGAGTCCGGTGA-3′	This study
2	5′-TTCTCGGAGGCCGTGCCATCGCATCTA-3′	This study
3	5′-GGATCCGTGAGCGAACTGCGGTTG-3′	This study
4	5′-CATATGATCACGCTGAGCAGAATC-3′	This study

and the PCR product was cloned into pCR2.1 vector. The cloned *mca* gene was then excised by *Bam*HI and *Nde*I digestion and ligated with pALACE (De Smet *et al.*, 1999) previously digested with *Bam*HI and *Nde*I to create the plasmid pM4. Myco504 and wild-type *M. smegmatis* were transformed with pM4 and hygromycin/kanamycin-resistant transformants were selected. The transformants were screened by SDS-PAGE analysis and one transformant from each transformation was selected for further study; the transformants were named Myco504*mca* and WT*mca*.

To confirm that the expressed protein was the *mca* gene product, mycothiol amidase, MCA was purified from the wild-type *mca* strain which had been induced for 48 h with 1 % acetamide. The cells were harvested and the cell pellet was sonicated and purified using a TALON affinity resin (Clonetech) according to the manufacturer's instructions. The amidase activity of the purified protein was determined as described by Newton *et al.* (2000a).

Determination of mycothiol content during the growth cycle of mc^{2}155, Myco504 and Myco504*mshB*. Stock bacterial cultures of mc^{2}155, Myco504, Myco504*mshB* grown in TSB medium were diluted to OD_{600} 0·05 with TSB medium supplemented with appropriate antibiotics. The cultures were diluted in 1 l Erlenmeyer flasks and incubated at 37 °C. The OD_{600} was measured at various time intervals with a Beckman DU 640 spectrophotometer using a cuvette with a pathlength of 1 cm. Samples of the cultures were harvested at various time points. Cells were collected by centrifugation and frozen for later mycothiol analysis. This experiment was repeated three times.

Determination of mycothiol content of Myco504 *mca*. Stock bacterial cultures of mc^{2}155, Myco504 and Myco504*mca* grown in Middlebrook medium supplemented with OADC were diluted to OD_{600} 0·5 and then washed twice with Middlebrook medium without OADC to remove the OADC. The cultures were divided into two flasks, one containing Middlebrook 7H9 medium with 1 % glucose and one containing Middlebrook 7H9 medium with 1 % acetamide for the induction of the acetamidase promoter in the pALACE vector. Samples were taken at 0, 24 and 48 h from both flasks and the mycothiol content of the cells was determined as above.

RESULTS

Creation and isolation of an *mshB* mutant of *M. smegmatis*

An 870 bp ORF was identified in the uncompleted *M. smegmatis* genome sequence (http://www.tigr.org) by sequence comparison with ORF Rv1170. The *M. smegmatis* ORF encodes a protein of 290 aa while ORF Rv1170 encodes a protein of 303 aa. As seen in Fig. 1(a) the two proteins share 73 % amino acid similarity, suggesting that the *M. smegmatis* ORF encodes an *mshB* homologue.

As described in Methods, in order to create a MshB$^-$ mutant of *M. smegmatis*, cells were transformed with pHBJ280 and kanamycin-resistant, streptomycin-sensitive transformants were isolated. To identify mutants that had undergone replacement of the wild-type *mshB* gene with the disrupted version, Southern hybridization was performed on *Eco*RI/*Eco*RV-digested genomic DNA from parent and putative mutant strains. The DIG-labelled hybridization probe corresponded to the sequence from 177 bp upstream of the *mshB* gene and first 176 bp of the *mshB* ORF. As seen in Fig. 1(b, c), the probe hybridized to a 12 kb *Eco*RI fragment in the parent strain. Disruption of *mshB* by the *aph* gene introduced an *Eco*RV site into the 12 kb *Eco*RI fragment which resulted in hybridization to a 7 kb fragment in the mutants. Three of the putative mutants had a characteristic hybridization pattern suggesting that the *mshB* gene had been replaced by the disrupted version. One such mutant, Myco504, was chosen for further analysis.

Myco504 contains low levels of mycothiol

Myco504 was analysed for mycothiol content to determine whether disruption of *mshB* affected mycothiol synthesis. In the exponential phase of growth, Myco504 contained 5–10 % of the wild-type levels of mycothiol: $1{\cdot}00 \pm 0{\cdot}21$ μmol (g dry weight)$^{-1}$ as compared with $11{\cdot}07 \pm 0{\cdot}58$ μmol (g dry weight)$^{-1}$ for *M. smegmatis* wild-type mc^{2}155. As seen in Fig. 2, the amount of mycothiol in the parent strain was dependent on the phase of growth of the cultures and ranged from 1·00 to 3·7 nmol per OD_{600} unit ml^{-1}, whereas Myco504 consistently had low levels of mycothiol (0·15 to 0·249 nmol per OD_{600} unit ml^{-1}). The parent strain and Myco504 grew at similar rates in TSB and Middlebrook 7H9 media, indicating that disruption of *mshB*, which causes a decrease in mycothiol levels, still permits normal growth under these conditions (Fig. 3).

Complementation of Myco504 with *M. smegmatis mshB* restores parental mycothiol levels

To confirm that the decrease in mycothiol was caused by the disruption in *mshB*, *mshB* was reintroduced into Myco504. The *M. smegmatis mshB* ORF and 300 bp of

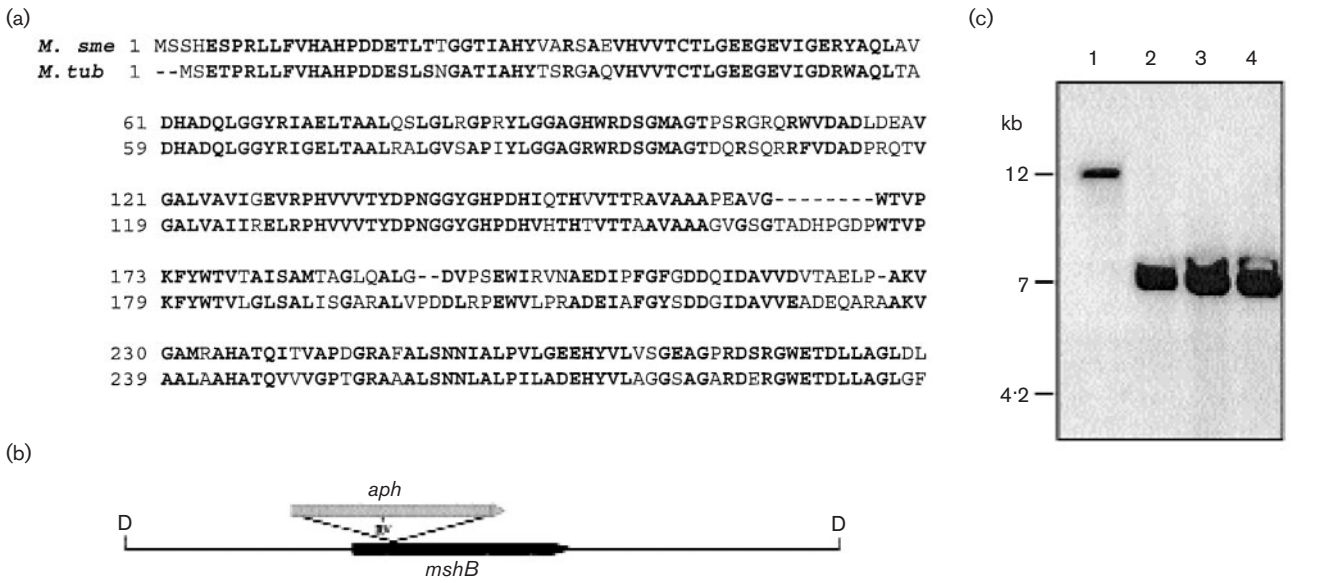

Fig. 1. Sequence comparison and Southern analysis of *mshB* inactivation in *M. smegmatis*. (a) Amino acid sequence alignment of mtMshB and msMshB. Identical and similar amino acids are in bold. (b) Schematic representation of *mshB* inactivation in *M. smegmatis* and the site of disruption of the Myco504 MshB$^-$ mutant. RV, *Eco*RV site. (c) Southern blot of *Eco*RI/*Eco*RV-digested genomic DNA from (1) wild-type and (2–4) mutants with disrupted *mshB*. The blot was probed with DIG-labelled DNA corresponding to the 5′ region of *mshB*. The probe hybridized to a 12 kb *Eco*RI fragment in the wild-type strain. The disruption of *mshB* in the mutants introduced an *Eco*RV site into the fragment which resulted in the probe hybridizing to a ~7 kb fragment in the mutant strains.

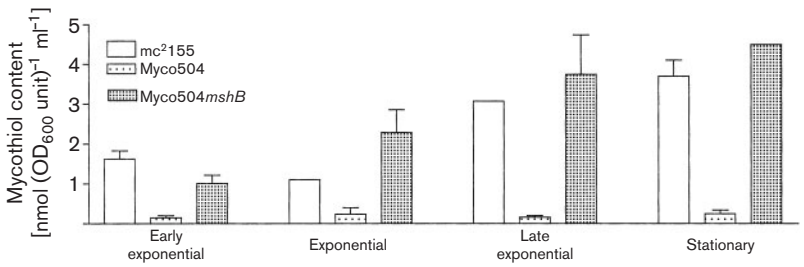

Fig. 2. Mycothiol contents of mc²155, Myco504 and Myco504*mshB* during different growth phases. Growth phases were according to the OD_{600} as determined from growth curves for: early exponential phase 0·5–1·0, exponential phase 1·0–4·0, late exponential phase 4·0–5·0, and stationary phase 5·0–7·0. The error bars denote SE.

upstream sequence were cloned into *M. smegmatis–E. coli* pGINT integrative vector. Nine transformants were sampled during exponential-phase growth and assayed for mycothiol content. All transformants had mycothiol levels of approximately 11 μmol g^{-1}. One transformant, Myco504-*mshB*, was further analysed for mycothiol content during the various phases of its growth cycle (Fig. 2). Myco504-*mshB* grew at essentially the same rate as mc²155 and Myco504 (Fig. 3) and introduction of *mshB* restored mycothiol levels.

Expression of *M. tuberculosis mca* in Myco504 does not increase mycothiol levels

MshB was shown to have weak mycothiol amidase (MCA) activity (Newton *et al.*, 2000b). However, whether MCA has mycothiol deacetylase activity has not been conclusively verified *in vivo*. MCA and MshB have similar amino acid sequences (36 % identity in 299 aa overlap) and thus we hypothesized that MCA may have some deacetylase activity that is responsible for the basal level of mycothiol present in the MshB⁻ mutant. To examine this hypothesis, we cloned the *M. tuberculosis mca* gene in the *M. smegmatis–E. coli* shuttle vector pALACE and named it pM4. In this vector, the cloned gene was under the control of an inducible acetamidase promoter and also had the added advantage of a hexahistidine tag allowing easy purification of the expressed protein. Both Myco504 and the wild-type parent *M. smegmatis* were transformed with pM4 and the transformants screened by SDS-PAGE analysis after induction with acetamide to overexpress *mca* in the MshB⁻ mutant as well as the parental strain. SDS-PAGE clearly showed that *mca* is overexpressed in Myco504*mca*, the mutant strain with the overexpressed *mca*, and in WT*mca*, the parent strain with the overexpressed *mca* (Fig. 4). To confirm that the induced protein was enzymically active, recombinant MCA was purified from the induced WT*mca* cell extract using affinity chromatography as above (Fig. 4) and checked for amidase activity as described by Newton *et al.* (2000a). The purified MCA had activity levels comparable to the purified native *M. tuberculosis* protein (G. Newton, personal communication), indicating that the recombinant MCA is indeed active. Mycothiol levels were then determined for induced and uninduced Myco504*mca*. We found no difference in the levels of mycothiol in Myco504 and Myco504*mca*, indicating that under the growth conditions we have tested, MCA is not capable of increasing mycothiol levels in the mutant (data not shown).

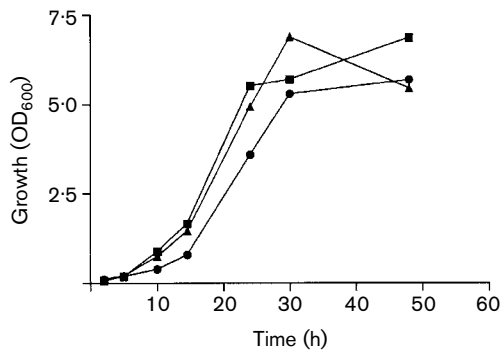

Fig. 3. Growth curves of parental strain mc²155 (■), Myco504 mutant (▲) and Myco504*mshB*-complemented mutant (●).

Sensitivity to antibiotics, oxidative stress and toxins

We previously reported that mutants lacking mycothiol are more susceptible to a broad range of oxidizing and alkylating agents (Rawat *et al.*, 2002). To determine whether the MshB⁻ mutant, Myco504, was also sensitive to these agents, we performed disk diffusion assays using a range of chemicals. Myco504 and wild-type *M. smegmatis* were not significantly different in sensitivity to redox cycling agents and cumene hydroperoxide (Table 2). However, Myco504 was more sensitive to mBBr, a fluorescent alkylating agent routinely used for thiol analysis, and CDNB, a glutathione-*S*-transferase substrate. Furthermore, Myco504 did not exhibit increased sensitivity to iodoacetamide and DTNB, two toxins that specifically react with the –SH bond (Table 2).

The MshB⁻ mutant is sensitive to ethionamide

Since mycothiol mutants were previously shown to be more sensitive to several antibiotics (Rawat *et al.*, 2002) and hyper-resistant to isoniazid (Newton *et al.*, 1999; Rawat *et. al.*, 2002; Koledin *et al.*, 2002; Newton *et al.*, 2003) we tested the antibiotic sensitivity of Myco504. In contrast to mutants lacking MshC ligase activity, Myco504 did not exhibit any significant difference in sensitivity to cerulenin, vancomycin, rifampicin and erythromycin as measured by disk diffusion assays (Table 3) and/or E-strips (data not

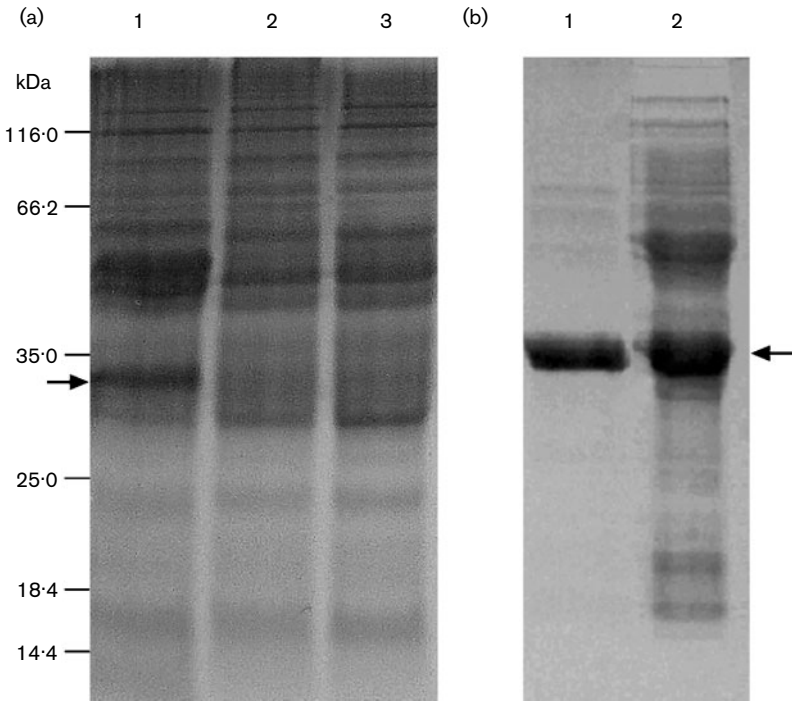

Fig. 4. SDS-PAGE analysis of overexpression of MCA in WT*mca* and Myco504*mca*. Ten micrograms of protein were loaded in each lane. (a) Sonicates of: lane 1, Myco504*mca* induced with 1 % acetamide for 48 h; lane 2, Myco504 *mca* grown in 1 % glucose; lane 3, mc^{2}155 grown in 1 % glucose. (b) Lane 1, purified protein from His-tag column; lane 2, sonicate of WT*mca* induced with 1 % acetamide for 48 h. The arrows indicate the induced MCA protein.

shown). Moreover, the MIC of isoniazid for the mc^{2}155 parent strain and Myco504 MIC, as measured by the agar dilution method, was the same (1–12·5 μg ml^{-1}). In contrast to these antibiotics, testing for ethionamide sensitivity revealed that the MIC for ethionamide of Myco504 (150 μg ml^{-1}) was sixfold higher than that of the parental wild-type *M. smegmatis* strain (25 μg ml^{-1}). Since we had reported previously that ethionamide

Table 2. Responses of growth of mc^{2}155 and Myco504 (MshB$^-$ mutant) to various oxidative stressors and toxins

Agent	Amount (μmol)	Zone of inhibition (diameter, mm)*	
		mc^{2}155	Myco504
Cumene hydroperoxide	0·1	13·0±0·0	12·5±0·5
	1·0	33·0±1·0	32·0±0·0
	5·0	51·0±1·0	53·0±1·0
Nitrofurantoin	1·0	18·0±2·0	17·5±2·5
	10·0	20·5±1·5	21·5±0·5
Menadione	0·1	15·0±1·0	19·0±3·0
	1·0	40·0±0·0	42·0±2·0
Plumbagin	0·01	22·0±2·0	29·0±3·0
	0·1	40·0±2·0	40·0±0·0
	1·0	67·0±1·0	63·0±7·0
MBBr	0·5	9·0±0·0	9·5±0·5
	1·0	18·5±0·5	20·5±0·5
	5·0	34·0±1·0	39·0±1·0
CDNB	0·1	18·0±1·0	20·0±0·0
	1·0	45·0±1·0	64·0±2·0
Iodoacetamide	0·01	14·0±1·0	15·0±2·0
	0·1	40·0±2·0	42·0±0·0
DTNB	0·01	12·0±1·0	13·0±0·0
	0·1	19·5±1·5	21·5±0·5
	1·0	20·5±1·5	21·5±0·5

*Means ± SE.

Table 3. Antibiotic responses of mc²155 and Myco504 (MshB⁻ mutant) as determined by disk assays

Agent	Amount (μg)	Zone of inhibition (diameter, mm)*	
		mc²155	Myco504
Cerulenin	0·5	14·0±1·0	14·5±0·5
	1·0	20·0±0·0	19·5±0·5
Vancomycin	5·0	15·0±0·0	15·0±0·0
	25·0	21·5±1·5	20·0±0·0
	50·0	24·5±0·5	23·0±0·0
Rifampicin	8·0	11·5±0·5	9·0±1·0
	32·0	20·0±1·0	18·5±1·5
Erythromycin	32·0	22·0±3·0	19·0±2·0

*Means ± SE.

sensitivity is not affected in MshC⁻ mutants lacking mycothiol (Rawat *et al.*, 2002), we examined all mutants available to us for their sensitivity to ethionamide. As seen in Table 4, mutants A1 and 49, which are both deficient in mycothiol production due to the inactivation of *mshA* (Newton *et al.*, 2003), are ethionamide resistant. On the other hand, as we reported earlier, MshC⁻ mutant I64 exhibits sensitivity to ethionamide identical to wild-type *M. smegmatis*.

DISCUSSION

Here, we report the disruption of *mshB*, a key enzyme in mycothiol biosynthesis. We show that despite the disruption, the mutant strain is still able to synthesize mycothiol, although at a lower level which does not exceed 5–10 % of the parental *M. smegmatis* mycothiol level. Reintroduction of the *mshB* gene into the mutant restored mycothiol levels and complemented the MshB⁻ mutant, indicating that *mshB* is indeed responsible for the synthesis of the majority of the mycothiol in *M. smegmatis*. Inhibition of MshB by mycothiol appears to be a major factor regulating mycothiol biosynthesis. Newton *et al.* (2000b) showed that GlcNAc-Ins accumulates in *M. smegmatis* despite the presence of substantial deacetylase activity in the cells whereas GlcN-Ins and Cys-GlcN-Ins levels are quite low. This implicates MshB activity as a control point regulating mycothiol production.

Table 4. Sensitivity of various mycothiol-deficient mutants to ethionamide

Strain	Zone of inhibition* (mm)	
	50 μg eth.	150 μg eth.
mc²155	34±0·0	54·0±0·0
Myco504	0	0
Myco504*mshB*	19±1·0	34±0·0
Myco504*mca*	0	0
A1(MshA⁻)	0	0
A1c	0	23±1·0
49 (MshA⁻)	0	0
49c	0	23±1·0
I64 (MshC⁻)	33±1·0	52±1·0

*Mean diameter (±SE) of zone of inhibtion around paper disks impregnated with 50 μg and 150 μg of ethionamide (eth.).

That a mutant in mycothiol biosynthesis still contains observable levels of mycothiol is not unprecedented. Previously, we reported a chemical missense mutant in *mshC* that retained 1–5 % of the mycothiol of the parental strain (Rawat *et al.*, 2002). Nevertheless, the levels of mycothiol in the MshB⁻ mutant are the highest reported so far for a mutant missing a gene in mycothiol biosynthesis. Indeed, the other mutants in *mshA* and *mshD* have virtually no mycothiol (*mshA*: Newton *et al.*, 1999, 2003; *mshD*: Koledin *et al.*, 2002).

Earlier we reported that MshB can act *in vitro* as both a deacetylatse and an amidase like MCA (Newton *et al.*, 2000b). However, the deacetylation activity of MCA has not been checked *in vivo* and it could be that it is MCA that is responsible for the basal levels of mycothiol in the *mshB*⁻ mutant. Overexpression of *mca* in the MshB⁻ mutant did not result in an increase in mycothiol levels. It is entirely possible that MCA is not responsible for the mycothiol present in the MshB⁻ mutant. Alternatively, it is also possible that even a small amount of mycothiol as in the MshB⁻ mutant may downregulate or block MCA activity. In that case, overexpressing MCA may not have any observable effect on the mycothiol content of the mutant. A double mutant, MshB⁻ Mca⁻, may reveal whether it is indeed the MCA that is responsible for the 5–10 % mycothiol that is present in Myco504.

Recently, Vetting *et al.* (2002) reported the crystal structure of an *M. tuberculosis* aminoglycoside 2′-*N*-acetyltransferase, AAC(2′), that catalyses the coenzyme A (CoA)-dependent acetylation of the 2′-hydroxyl or amino group of a wide range of aminoglycosides. The authors hypothesize that this enzyme may acetylate GlcN-Ins to form GlcNAc-Ins in a reverse reaction to MshB deacetylase. It is unlikely that this enzyme catalyses the reverse reaction for two main reasons: (i) if GlcN-Ins acetylation activity was present in *M. smegmatis* we would not been able to isolate the *mshC* mutants with high levels of GlcN-Ins as described in our previous publication (Rawat *et al.*, 2002); and (ii) AAC(2′) lacks any metal ion, which is required for hydrolytic activity of MshB.

In our recent publication we reported that mutants lacking mycothiol and MshC ligase activity are more sensitive to antibiotic, oxidative and alkylating stress (Rawat *et. al.*, 2002). Interestingly, the *mshB* mutant, Myco504, did not display increased sensitivity to oxidative stresses and to most alkylating agents with the exception of mBBr and

CDNB. Most surprising was the finding that the *mshB* mutant is not resistant to the prodrug isoniazid. *M. smegmatis* mc^{2}155 and the MshB$^-$ mutant derived from it are equally sensitive to isoniazid (MIC 1–12·5 μg) whereas MshA$^-$ mutants and the MshD$^-$ mutant are extremely resistant to isoniazid (MIC $\geqslant$256 μg) (Newton *et al.*, 1999, 2003; Koledin *et al.*, 2002). Even mutants with mycothiol levels that are 1–5 % of the wild-type amount, such as the MshC$^-$ mutant I64, are isoniazid resistant, having a MIC of 32 μg that is significantly higher than the MIC of the wild-type *M. smegmatis*. Mycothiol is normally present in millimolar levels in the wild-type cell; however, the amount of mycothiol present in Myco504 appears to be sufficient to maintain isoniazid sensitivity and protect the cells against oxidative stress, some alkylating stress, and antibiotic stress under otherwise normal growth conditions.

Possibly the most exciting finding from this work is the drug resistance of the mutants. Ethionamide and isoniazid are both specific antimycobacterial drugs that share at least one site of action in mycolic acid biosynthesis. Isoniazid is a prodrug which is oxidized by the bacterial catalase-peroxidase, KatG, to form a reactive toxic species. Mutations in the KatG gene that confer isoniazid resistance do not result in ethionamide resistance. Despite the common site of action, ethionamide and isoniazid are activated by different enzymes. The first step of activation of ethionamide is an NADPH- and O_2-dependent reaction that yields the corresponding *S*-oxide metabolite which requires further activation to a final cytotoxic species (Vannelli *et al.*, 2002; De Barber *et al.*, 2000). We have compared the ethionamide sensitivity of *M. smegmatis* mc^{2}155 and MshA$^-$, MshB$^-$ and MshC$^-$ mutants derived from it. The MshA$^-$ and MshB$^-$ mutants were resistant to ethionamide. Complementation of the MshB$^-$ mutant restored wild-type sensitivity to ethionamide; however, after *mshA* complementation of the MshA$^-$ mutants the strains were still resistant to low levels of ethionamide (Table 4). These results are in contrast to the MshC$^-$ mutant, I64, which was as sensitive to ethionamide as wild-type *M. smegmatis*. Because resistance to ethionamide is not a characteristic of all mutants that lack mycothiol, it is most likely that an intermediate in the mycothiol biosynthetic pathway but not mycothiol itself is involved in ethionamide resistance. This effect could be indirect, occurring through the regulation or activation of enzymes that partcipate in ethionamide activation.

Baulard *et al.* (2000) have demonstrated that expression of EthA, the flavin monooxygenase which activates ethionamide in mycobacteria, is controlled by a regulator, EthR, which itself may be regulated. When EthR is expressed at high levels, EthA is repressed, resulting in ethionamide resistance. The EthA/EthR system does not modulate resistance to isoniazid. The mechanism of EthR regulation is unknown; however, it is possible that EthA is repressed in the MshA$^-$ and MshB$^-$ mutants and not in the MshC$^-$ mutant. Futher investigation to test this hypothesis is under way.

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