Involvement of the N- and C-terminal domains of *Mycobacterium tuberculosis* KatG in the protection of mutant *Escherichia coli* against DNA-damaging agents

Michelle A. Mulder,¹ Shamila Nair,^{1,2} Valerie R. Abratt,³ Harold Zappe¹ and Lafras M. Steyn¹

Author for correspondence: Lafras M. Steyn. Tel: +27 21 4066389. Fax: +27 21 4488153.
e-mail: lsteyn@medmicro.uct.ac.za

¹ Department of Medical Microbiology, University of Cape Town and Groote Schuur Hospital, Medical School, Observatory, 7925 Cape Town, South Africa

² Faculté de Médecine Necker-Enfants malades, Laboratoire de Microbiologie, 156, rue de Vaugirard, 75730 Paris Cedex 15, France

³ Department of Microbiology, University of Cape Town, Rondebosch, 7800 Cape Town, South Africa

The *Mycobacterium tuberculosis* KatG enzyme, like most hydroperoxidase I (HPI)-type catalases, consists of two related domains, each with strong similarity to the yeast cytochrome c peroxidase. The catalase-peroxidase activity is associated with the amino-terminal domain but currently no definite function has been assigned to the carboxy-terminal domain, although it may play a role in substrate binding. This paper reports another possible function of the KatG protein involving protection of the host cell against DNA-damaging agents. The *M. tuberculosis* katG gene, the 5' domain and the 3' domain were cloned separately, in-frame with the maltose-binding protein, into the vector pMAL-c2. These constructs were introduced into four DNA-repair mutants of *Escherichia coli*, DK1 (*recA*), AB1884 (*uvrC*), AB1885 (*uvrB*) and AB1886 (*uvrA*), which were then tested for their ability to survive treatment with UV light (254 nm), hydrogen peroxide (1.6 mg ml⁻¹) and mitomycin C (6 µg ml⁻¹). All three constructs conferred resistance to UV upon the *recA* *E. coli* cells, whereas resistance to mitomycin C was found in all repair mutants tested. Protection against hydrogen peroxide damage was less pronounced and predominantly found in the *recA* host. These results indicated that the *M. tuberculosis* katG gene can enhance DNA repair in *E. coli*, and that the 5' and 3' domains can function separately. UV sensitivity tests on *Mycobacterium intracellulare* and *M. tuberculosis* strains mutant in katG revealed that the katG gene product does not play an additive role in the survival of mycobacterial cells after exposure to short-wavelength UV irradiation, in repair-competent cells.

Keywords: *Mycobacterium tuberculosis*, KatG, catalase, DNA repair

INTRODUCTION

Mycobacterium tuberculosis is an intracellular pathogen that survives host macrophage bacterial killing mechanisms, including DNA-damaging agents such as peroxide and nitric oxide. The *M. tuberculosis* katG gene

encodes a dual-function catalase-peroxidase enzyme which protects the organism against hydrogen peroxide and thus contributes to its survival in macrophages (Middlebrook & Cohn, 1953; Mitchison *et al.*, 1960; Jackett *et al.*, 1978; Wilson *et al.*, 1995; Heym *et al.*, 1997). *M. tuberculosis* KatG also plays an important role in susceptibility of the organism to the front-line antituberculosis drug, isoniazid (INH) (Middlebrook, 1954; Winder, 1960; Zhang *et al.*, 1992; Heym *et al.*, 1993; Stoeckle *et al.*, 1993; Altamirano *et al.*, 1994; Rouse & Morris, 1995).

Like most hydroperoxidase I (HPI)-type catalases, the

Abbreviations: ADC, albumin dextrose catalase; EMS, ethyl methane-sulfonate; HPI, hydroperoxidase I; INH, isoniazid; MBP, maltose-binding protein.

The GenBank/EMBL/DDJB accession number for the sequence reported in this paper is L14268.

M. tuberculosis KatG enzyme consists of two related domains, each with strong similarity to the yeast cytochrome *c* peroxidase. Both the catalase and peroxidase activities are associated with the amino-terminal domain (Welinder, 1991, 1992), but currently no definite function has been assigned to the carboxy-terminal domain, although it may play a role in substrate binding. Deletion of the 3' end of the gene results in loss of enzyme activity and in resistance to INH (Zhang *et al.*, 1992).

Repair of damaged DNA is also expected to be an important factor involved in the survival of mycobacteria in macrophages. DNA repair has been extensively studied in *Escherichia coli* and found to be complex, involving a number of specialized proteins and pathways, including the SOS response and genetic recombination (Friedberg *et al.*, 1995). However, relatively little is known about these systems in mycobacteria. It has been shown that there is an SOS response system active in *M. tuberculosis* (Durbach *et al.*, 1997; Movahedzadeh *et al.*, 1997a, b; Papavinasundaram *et al.*, 1997). The *lexA* repressor has been implicated in the induction of a *recA* gene in *Mycobacterium smegmatis* (Durbach *et al.*, 1997). The *M. tuberculosis* genome data have recently been analysed with respect to genes involved in DNA repair (Mizrahi & Andersen, 1998); homologues for nucleotide excision repair, base excision repair and SOS repair and mutagenesis were present. Notably, however, genes involved in mismatch repair were absent.

The *recA* genes from a number of mycobacteria have been cloned and sequenced (Davis *et al.*, 1991). These genes have an unusual structure in the major mycobacterial pathogens, *Mycobacterium leprae* and *M. tuberculosis*. In these organisms the genes encode an additional protein element, an intein, which is spliced out after translation to produce the mature, functional protein (Davis *et al.*, 1991, 1994). The protein, although it reacts with antibodies to the *E. coli* RecA, differs from the latter protein in certain properties. The *M. tuberculosis* protein is defective in displacing single-stranded binding protein (SSBP) from ssDNA, and has a reduced affinity for, and reduced ability to hydrolyse, ATP. It also shows significant differences in its requirements for binding to DNA, and in the formation of joint molecules (Davis *et al.*, 1991).

Another important determinant involved in the survival of cells exposed to agents that cause oxidative damage is the *oxyR* gene. In *M. tuberculosis*, this gene is naturally mutated (Deretic *et al.*, 1995; Sherman *et al.*, 1995; Dhandayuthapani *et al.*, 1996). This, together with the unusual nature of the *recA* gene, suggests that the pathways and mechanisms of DNA repair in this organism may differ from those in other, well-characterized bacteria. These unusual features may also contribute to the virulence of *M. tuberculosis*.

We report here on the involvement of the *M. tuberculosis katG* gene in protection of DNA-repair-

deficient *E. coli* mutants against DNA-damaging agents. Heterologous hosts were used since there are no defined *M. tuberculosis* DNA-repair mutants. The *M. tuberculosis katG* gene, the 5' domain and the 3' domain were cloned separately, in-frame with the maltose-binding protein, in the vector pMAL-c2 (New England Biolabs). These constructs were introduced into four different DNA-repair mutants of *E. coli*, which were tested for their ability to survive exposure to UV light, hydrogen peroxide and mitomycin C.

METHODS

Bacterial strains, culture conditions and transformation methods. The source and characteristics of the strains used in this study are summarized in Table 1. *E. coli* UM255 was used as the cloning host and for the analysis of enzyme activity. *E. coli* cells were grown in 2YT broth (Miller, 1972) at 37 °C with shaking and plated on 2YT agar plates. The cells were made competent using dimethyl sulfoxide (Chung & Miller, 1988), and transformants were plated on agar plates containing 50 µg ampicillin ml⁻¹ and, where necessary, 0.3 mM IPTG and 100 µg X-Gal ml⁻¹. The mycobacterial strains were grown in Middlebrook 7H9 medium supplemented with albumin, dextrose, catalase (ADC) and Tween 80, and plated on Middlebrook-ADC agar plates containing 0.01 % (w/v) cycloheximide.

DNA manipulations. DNA isolation and manipulations were carried out using standard techniques (Sambrook *et al.*, 1989). Sequencing of double-stranded DNA was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the Sequenase 2.0 (USB) and Taq Track (Promega) kits. DNA templates for sequencing were prepared by progressive deletions of double-stranded template DNA with exonuclease III (BRL) (Henikoff, 1987). Custom-synthesized oligonucleotides were used to prime the reactions and the sequence of the insert was analysed using GCG (Genetics Computer Group, 1991) and GENPRO version 6.10 (Riverside Scientific Enterprises). Searches for nucleotide and amino acid sequence similarities were done using the BLAST program (Altschul *et al.*, 1990) in the EMBL database.

Construction and screening of a DNA library. The construction of the *Pst*I genomic library of *M. tuberculosis* H37Rv in the suicide vector pEcoR252 was described previously (Nair & Steyn, 1991). The library was screened by selection of transformed *E. coli* DK1 cells on plates containing 0.1 % (w/v) ethyl methane sulfonate (EMS).

Cloning of the truncated *M. tuberculosis katG* gene in pMAL-c2. The 1747 bp *Pst*I fragment from the DNA library clone, pD3, was cloned into the *Pst*I site of the expression vector, pMAL-c2 (New England Biolabs), to form pSMK3' (Fig. 1). Use of this restriction endonuclease results in an in-frame fusion of the terminal third of the *M. tuberculosis katG* gene with the maltose-binding protein (MBP), encoded by *malE*. During cloning, a construct was isolated which contained the 1747 bp insert in the opposite orientation to that in pSMK3'. This construct was designated pSMK3'_{opp}. The reading frame of the *M. tuberculosis katG* gene, with respect to the *malE* gene, was altered using the following strategy. pSMK3' was digested with *Bam*HI, which restricts the construct upstream of the insert. The resulting cohesive ends were removed by digestion with mung bean nuclease (Boehringer Mannheim), and the blunt-ended, linear fragment was religated to form the

Table 1. Bacterial strains used in this study

Strain	Genotype	Reference
<i>E. coli</i>		
UM255	<i>pro leu rpsL hsdM hsdR endI lacY katG2 katE12::Tn10 recA</i>	Mulvey <i>et al.</i> (1988)
MC1060	<i>F⁻ hsdR mcrB araD139 Δ(ara leu)7697 galU galK lacX74 rpsL thi</i>	Meissner <i>et al.</i> (1987)
DK1	<i>Δ(srl-recA)306 Δ(ara leu)7697 lacX74 galU galK hsr araD139 strA</i>	ATCC 35691; Willis <i>et al.</i> (1981)
AB1157	<i>rac ara argE Δ(gpt-proA)62 galK hisG kdgK lacY1 leuB6 mgl51 mtl1 qsr rfbD1 rpsL supE44 thi1 thr1 tsx33 xyl5</i>	ATCC 29055; Howard-Flanders <i>et al.</i> (1966)
AB1884	As for AB1157 and <i>uvrC34</i>	Howard-Flanders <i>et al.</i> (1966)
AB1885	As for AB1157 and <i>uvrB5</i>	Howard-Flanders <i>et al.</i> (1966)
AB1886	As for AB1157 and <i>uvrA6</i>	Howard-Flanders <i>et al.</i> (1966)
Mycobacteria		
<i>M. intracellulare</i> 1403 <i>katG</i> ⁺	<i>katG</i> ⁺ parent	B. Marklund, Stockholm, Sweden
<i>M. intracellulare</i> 1403 <i>katG</i> ⁻	<i>katG</i> mutant	B. Marklund, Stockholm, Sweden
<i>M. tuberculosis</i> 34	INH-resistant clinical isolate; <i>ΔkatG</i>	Heym <i>et al.</i> (1994)
<i>M. tuberculosis</i> 255	INH-susceptible clinical isolate; no detectable mutations in <i>katG</i>	T. C. Victor, University of Stellenbosch, SA
<i>M. tuberculosis</i> 463	INH-resistant clinical isolate; mutations in codons 463 and 315 of <i>katG</i>	T. C. Victor, University of Stellenbosch, SA
<i>M. tuberculosis</i> 519	INH-resistant clinical isolate; mutation in codon 315 of <i>katG</i>	T. C. Victor, University of Stellenbosch, SA

Fig. 1. Linear restriction map of the *M. tuberculosis katG* gene and downstream sequences. The *EcoRI* and *XbaI* sites were introduced by PCR. The positions of the *katG* coding region and the two downstream ORFs are indicated. The regions present in the pMAL-c2 constructs are shown.

construct pSMK3'a, in which the *katG* sequences are translated in frame 2. The construct pSMK3'b was made by digestion of pSMK3' with *SalI*, which digests the construct upstream of the insert, filling in of the resulting cohesive ends using T4 DNA polymerase (Amersham DNA Blunting kit), and religation of the blunt-ended, linear fragment. In this construct, the *katG* sequences are translated in frame 3. Another construct, pSMKd, was made by digestion of the 1747 bp fragment with *AluI* and *PstI*, to generate a 961 bp fragment, containing the sequences downstream of the *M. tuberculosis katG* gene. The fragment was cloned into the

XmnI and *PstI* sites of pMAL-c2 to yield pSMKd (Fig. 1). The orientation and frame of the inserts of these constructs were confirmed by restriction analysis and sequencing over the junctions using the *malE* primer (New England Biolabs), which initiates sequence near the 3' end of the *malE* gene, and the pUC19 forward primer (Promega).

PCR amplification and cloning of the complete *katG* gene in pMAL-c2. PCR amplification was performed (Saiki *et al.*, 1988) using *M. tuberculosis* H37Rv genomic DNA (0.25 µg) as the template with the synthetic primers 5'-AATGaatTcCCCGAGCAACACCCA-3', and 5'-GGCAGtctaGATC-

AACCCGAATCA-3', corresponding to the beginning and end of the *katG* gene, respectively. The lower-case nucleotides indicate mismatches and the underlined nucleotides represent the *EcoRI* and *XbaI* sites, respectively. The template DNA was denatured by heating to 95 °C for 5 min. The temperature profile of the amplification was as follows: 30 s at 94 °C, 60 s at 60 °C and 90 s at 72 °C for 36 cycles, followed by further incubation at 72 °C for 5 min. Amplified PCR products were separated on a 1% (w/v) agarose gel and visualized by ethidium bromide staining and UV illumination. The 2.2 kb band was excised from the gel, purified using a GeneClean kit (Bio101) and cloned in the *EcoRI* and *XbaI* sites of pMAL-c2 to form pSMKA. A total of 1.1 kb of the insert was sequenced and was identical to the published *M. tuberculosis katG* sequence. The 3' end of the *katG* gene was deleted from pSMKA by digestion with *PstI*, to release a 726 bp DNA fragment, followed by religation to form the construct pSMK5'. The *PstI* fragment was also cloned into pMAL-c2, with the *katG* in-frame with *malE*, to form pSMK3' (Fig. 1).

SDS-PAGE and immunoblot analysis of proteins. Stationary-phase cultures of *E. coli* UM255 (*katG katE*) transformants harbouring pSMKA, pSMK5', pSMK3' and pMAL-c2 were diluted 100-fold into 5 ml fresh 2YT medium containing ampicillin and incubated at 37 °C with aeration (orbital shaker, 150 r.p.m.). When the cultures reached an OD₆₀₀ of 0.4, 1 ml of each was removed and the cells harvested by centrifugation. The cell pellets were resuspended in 50 µl SDS-PAGE sample buffer (Laemmli, 1970), and represented the uninduced control. IPTG (0.3 mM) was added to the remainder of the culture, and the cells were incubated for a further 2 h at 37 °C. These induced cells (1 ml) were harvested by centrifugation, and resuspended in 100 µl SDS-PAGE sample buffer. All samples were boiled for 5 min, loaded on a 12.5% (w/v) SDS-polyacrylamide gel, and separated by electrophoresis at a constant current of 40 mA. Proteins were visualized by staining with Coomassie Brilliant Blue R-250. For immunoblots, the proteins in unstained gels were transferred onto an Amersham C-super nitrocellulose membrane. Blots were probed with the anti-MBP rabbit serum supplied by New England Biolabs, and anti-rabbit IgG conjugated to peroxidase (POD) (Boehringer Mannheim). Immunostaining was performed using the Chemiluminescence Blotting Substrate (POD) (Boehringer Mannheim).

Catalase and peroxidase activity assays. *E. coli* UM255 (*katG katE*) cells harbouring the pMAL-c2 constructs were grown and induced with IPTG, as described above. The cells were collected by centrifugation, and resuspended in 500 µl 0.1 M potassium phosphate buffer (pH 7.0) and 500 µl lysozyme (4 mg ml⁻¹). The cell suspensions were incubated at 37 °C for 60 min and frozen at -20 °C overnight. The extracts were thawed in ice-water, and the cellular debris was removed by centrifugation. The supernatants were recovered, and the pellets were suspended in 1 ml 0.1 M potassium phosphate buffer, pH 7.0. The protein concentrations of the supernatant and pellet fractions were determined using the Bio-Rad Protein Assay kit and BSA standards. Gel-staining catalase and peroxidase activity assays were performed by the methods of Clare *et al.* (1984) and Wayne & Diaz (1986). Briefly, 70 µg each of the supernatant and pellet fractions were separated on 7.5% (w/v) non-denaturing polyacrylamide gels. For catalase stains, the gels were immersed in 5 mM hydrogen peroxide for 20 min, and then in a 1:1 solution of 2% ferric chloride and 2% potassium ferricyanide. Peroxide stains were performed by immersing the gels in a solution containing 5 mM hydrogen peroxide and 0.5 mg 3,3'-diaminobenzidine ml⁻¹. Catalase activity bands were visualized as clear bands on a dark green

background, and peroxidase activity bands as brown bands on a clear background.

UV sensitivity tests. IPTG-induced cultures of *E. coli* mutants transformed with the pMAL-c2 constructs were diluted 10-fold in water and irradiated with short-wavelength (254 nm) UV light at a fluence rate of 1 J m⁻² s⁻¹ using a single-wavelength lamp (model UVG-11 254 nm, UV Products). UV doses were measured using a model J-225 short-wavelength Blak-Ray UV meter (UV Products). Samples were withdrawn before irradiation and at the indicated times, diluted in water, and plated on 2YT agar containing ampicillin. After overnight incubation at 37 °C, colonies were counted and surviving fractions calculated relative to the unirradiated sample. All experiments were performed in the absence of direct light, and the plates were incubated in the dark to avoid photoreactivation (Jones & Woods, 1981; Abratt *et al.*, 1985). Mycobacterial cells were grown to stationary phase in 5 ml Middlebrook-ADC-Tween medium. Irradiated cells were plated on Middlebrook-ADC agar plates containing 0.01% (w/v) cycloheximide and incubated at 37 °C for 4–6 weeks. Clumping of the mycobacterial cells was minimized by including Tween 80 in the medium, and by vigorous stirring of the cell suspension before diluting and plating. All experiments were performed in triplicate. Preliminary experiments were performed to determine the doses of UV irradiation which resulted in a significant difference between the survival curves of parent and mutant strains, but which allowed sufficient survival of mutants so that colony counts remained accurate.

Mitomycin C sensitivity tests. IPTG-induced cultures (as described above) were diluted 10-fold in fresh medium and a sample was removed for plating. Mitomycin C (Merck) was added to a final concentration of 6 µg ml⁻¹ and the cells were incubated at 37 °C with shaking. Samples were removed at different time points, diluted in water and plated. Colonies were counted after overnight incubation at 37 °C and surviving fractions calculated as described for the UV sensitivity experiments. Preliminary experiments were performed to determine the appropriate dose of mitomycin C for use in the above experiments.

Hydrogen peroxide sensitivity tests. Hydrogen peroxide tests were carried out by the method of Demple *et al.* (1983), as described for mitomycin C except that hydrogen peroxide was added to a final concentration of 1.6 mg ml⁻¹. Results were processed in the same way as for the UV and mitomycin C experiments. Preliminary experiments were performed to determine the appropriate dose of hydrogen peroxide for use in the above experiments.

RESULTS

Isolation and sequencing of a clone that confers resistance to EMS upon *E. coli* DK1

A *PstI* genomic library of *M. tuberculosis* H37Rv in the suicide vector pEcoR252, a derivative of pEcoR251 (Zappe *et al.*, 1986), was screened for DNA-repair genes by selection of *E. coli* DK1 (*recA*) transformants on 0.1% (w/v) EMS. Two clones were isolated that were able to survive this treatment: one, encoding a putative *recA* gene, was described earlier (Nair & Steyn, 1991) and the other, designated pD3, is described here. The 1747 bp insert from pD3 was sequenced (GenBank accession number L14268) and found to contain three possible ORFs, one of which was truncated at the 5' end.

Fig. 2. Expression of the MBP-KatG fusions in *E. coli* UM255. Total cellular proteins were separated on two 12.5% (w/v) SDS polyacrylamide gels. Proteins were visualized by staining with Coomassie blue (a) and by immunoblotting with anti-MBP serum (b). Lanes: 1, Molecular mass standards (Pharmacia); 2, untransformed *E. coli* UM255 induced; 3 and 4, uninduced and induced fractions from UM255(pMAL-c2); 5 and 6, corresponding fractions from UM255(pSMKA); 7 and 8, corresponding fractions from UM255(pSMK5'); 9 and 10, corresponding fractions from UM255(pSMK3'). The fusion proteins (F) are visible in lanes 4 (50 kDa MBP-LacZ), 6 (125 kDa MBP-KatGall and 90 kDa cleavage product, C), 8 (99 kDa MBP-KatG5' and 90 kDa cleavage product, C) and 10 (68 kDa MBP-KatG3').

A database search revealed that this ORF (241 amino acids) had 100% homology to the terminal portion of the *M. tuberculosis* *katG* gene and 55% to that of *E. coli* at the nucleotide level. The two downstream ORFs encode putative proteins of 168 and 56 amino acids, with no defined function (Fig. 1).

Fusion of *katG* with the MBP and overexpression in *E. coli*

In order to study this region of the *M. tuberculosis* *katG* gene further, the 1.7 kb insert from pD3 was cloned into the vector pMAL-c2 (New England Biolabs) with the

Fig. 3. Non-denaturing polyacrylamide gels stained for catalase (a) and peroxidase (b) activities. Supernatant and pellet fractions of IPTG-induced *E. coli* UM255 cells transformed with pMAL-c2, pSMKA, pSMK5' and pSMK3' were separated on 7.5% (w/v) non-denaturing polyacrylamide gels. Catalase and peroxidase stains were performed as described in Methods. Each lane contained 70 µg total protein. Lanes: 1 and 2, supernatant and pellet fractions from untransformed *E. coli* UM255; 3 and 4, the corresponding fractions from UM255(pMAL-c2); 5 and 6, corresponding fractions from UM255(pSMKA); 7 and 8, corresponding fractions from UM255(pSMK5'); 9 and 10, corresponding fractions from UM255(pSMK3'). *E. coli* UM255 is mutant in *katE* and *katG* and exhibits no native catalase or peroxidase activity. The only active fusion protein is that encoded by pSMKA.

truncated *katG* ORF in-frame with the MBP to form the construct pSMK3'. The complete *katG* gene was amplified by PCR using primers homologous to the 5' and 3' ends of the published sequence (GenBank/EMBL accession number X68081) and was also cloned in-frame with the MBP in pMAL-c2 to form the construct pSMKA. A third construct, pSMK5', was made by deleting the 726 bp *Pst*I fragment at the 3' end of the gene from pSMKA (Fig. 1). The vector pMAL-c2 contains the *malE* gene, encoding the MBP under control of the IPTG-inducible *tac* promoter. Expression of the fusion proteins is low under normal conditions due to the repressor encoded by the *lacIⁿ* gene. In the presence of IPTG, however, high levels of expression are obtained. Expression of the fusion proteins from the above constructs was tested by induction of *E. coli* UM255 (*katG katE*) cells carrying the plasmids and by electrophoresis of the resulting proteins on a 12.5% (w/v) SDS polyacrylamide gel (Fig. 2a). The fusion proteins encoded by pSMK3', pSMK5' and pSMKA were designated MBP-KatG3', MBP-KatG5' and MBP-KatGall, respectively. These proteins had relative mobilities of 68 kDa (MBP-KatG3'), 99 kDa (MBP-KatG5') and 125 kDa (MBP-KatGall), as predicted. The two larger fusion proteins, however, appeared predominantly (94.6% of MBP-KatGall and 92.8% of MBP-KatG5') as cleavage products of approximately 90 kDa. Both full-sized and cleavage products reacted with anti-MBP antibodies (Fig. 2b), suggesting that the cleavage site lies within the KatG portion of the fusion proteins. The presence and relative abundance of the cleavage products were independent of the *E. coli* host strain and the induction time. No discrepancies were observed in the nucleotide sequence of *katG* between nucleotides 3180

Fig. 4. Effect of the *katG* constructs on the survival of cells of *E. coli* DK1 (a, c, e) and *E. coli* AB1886 (b, d, f) exposed to (a, b) short-wavelength (254 nm) UV irradiation, (c, d) mitomycin C (6 $\mu\text{g ml}^{-1}$) and (e, f) hydrogen peroxide (1.6 mg ml^{-1}). Surviving fractions were calculated relative to the colony counts at time 0. The survival curves represent the mean of three experiments; error bars represent the standard error of the mean. The survival curves of the parent strains are shown in (a–d). ●, pMAL-c2; ■, pSMKA; ○, pSMK5'; □, pSMK3'; ▲, MC1060; △, AB1157.

and 3240 or codons 400 and 420 (numbered with respect to the published sequence, X68081), which lie 226–286 nucleotides upstream of the *Pst*I site, and are predicted to contain the cleavage site. This suggests that the

Table 2. Analysis of the sensitivities to UV, mitomycin C and hydrogen peroxide of *E. coli* DNA-repair mutants transformed with the *katG* constructs

Strain	Construct	Relative survival (x/c)*		
		UV	Mitomycin C	Hydrogen peroxide
DK1	pSMKA	220.3	8.84	30.89
	pSMK5'	210.6	23.05	19.99
	pSMK3'	108.7	24.86	0.33
AB1884	pSMKA	0.07	59.11	0.16
	pSMK5'	0.84	17.57	2.15
	pSMK3'	0.28	1.80	0.14
AB1885	pSMKA	0.62	6.91	4.31
	pSMK5'	0.98	16.29	0.002
	pSMK3'	1.08	9.16	0.003
AB1886	pSMKA	0.71	76.6	0.20
	pSMK5'	0.43	52.99	0.002
	pSMK3'	0.46	31.69	4.14

*Relative survival: the surviving fraction of a cell culture (x) harbouring a construct after 6 J m^{-2} (UV) or 12 min (mitomycin C and hydrogen peroxide) exposure, divided by the surviving fraction of the control culture, pMAL-c2 (c), after the same respective treatment. The relative survivals were calculated from the mean values of three experiments.

cleavage site was not introduced by an error in the coding sequence during the PCR amplification. The presence of cleavage products of the *M. tuberculosis* KatG has been noted by Johnsson *et al.* (1997) and Heym *et al.* (1997). In addition, proteolytic cleavage has been observed in the native catalase isolated from rat liver (Robbi & Lazarow, 1978; Crane *et al.*, 1982). Gel-staining catalase and peroxidase assays (Fig. 3) showed that only the fusion containing the whole KatG protein was active. This agrees with the results of Zhang *et al.* (1992), who demonstrated that deletion of the 3' end of the gene results in loss of enzyme activity. Activity therefore requires both domains of the protein and appears not to be affected by the presence of the MBP.

UV sensitivities of *E. coli* DNA-repair mutants carrying the *katG* constructs

Four repair-deficient, UV-sensitive *E. coli* strains, DK1, AB1884, AB1885 and AB1886, were used to test the effect of the KatG constructs. *E. coli* MC1060, the parent strain of DK1, and *E. coli* AB1157, the parent strain of AB1884, AB1885 and AB1886, were included as controls (Table 1). The repair-deficient *E. coli* strains harbouring the constructs pSMKA, pSMK5' and pSMK3' and the vector pMAL-c2 were examined for their ability to survive exposure to short-wavelength (254 nm) UV irradiation (Fig. 4a, Table 2). The surviving fraction of cells at a UV dose of 6 J m^{-2} was selected for comparative analysis. The parent *E. coli* strains MC1060

Fig. 5. Effect of UV irradiation on *E. coli* DK1 cells harbouring pSMK3' derivatives. The UV survival curves represent the mean of three experiments; error bars represent the standard error of the mean. Δ , DK1(pSMK3'); $\blacksquare$, DK1(pSMK3'*); $\blacktriangle$, DK1(pSMK3'a); $\circ$, DK1(pSMK3'b); $\square$, DK1(pSMKd); $\bullet$, DK1(pSMK3'_{opp}).

and AB1157 showed <1% killing at the UV doses used due to the presence of efficient DNA-repair systems. *E. coli* DK1(pMAL-c2) had a similar UV survival curve to that of untransformed *E. coli* DK1 (not shown). When *E. coli* DK1 was transformed with pSMKA or pSMK5', the cells exhibited a >200-fold increase in survival at the highest UV dose used (6 J m⁻²) (Fig. 4a, Table 2). A similar effect was observed with *E. coli* DK1(pSMK3'), with an increase in survival of 108-fold. The presence of the *katG* constructs, therefore, increased the UV resistance of the cells, although not to wild-type levels. From the *E. coli* AB1886 survival curves (Fig. 4b) and the values for AB1884 and AB1885 in Table 2, it is evident that none of the *katG* constructs conferred any resistance to UV upon the excision-repair mutants.

UV sensitivities of constructs containing various regions of pSMK3'

To determine whether the protective effects observed with the pSMK3' construct were due to the presence of the *M. tuberculosis* *katG*, or sequences downstream of the gene, *E. coli* DK1 cells harbouring the pSMK3' derivatives, pSMK3', pSMK3'a, pSMK3'b, pSMK3'_{opp} and pSMKd, were tested for their ability to survive exposure to short-wavelength UV irradiation. pSMK3'* contains only the *katG* portion of pSMK3', and no downstream sequences; pSMK3'a and pSMK3'b have bases either removed or added to change the frame of the insert with respect to the MBP; pSMK3'_{opp} contains the 1747 bp insert in the opposite orientation to that in pSMK3'; and pSMKd contains the region downstream of the *katG* gene (Fig. 1). Only the constructs pSMK3' and pSMK3'* express the MBP-KatG3' fusion protein and they alone conferred resistance to UV irradiation

upon the *E. coli* DK1 cells (Fig. 5). This property is independent of the presence of the downstream sequences.

Mitomycin C sensitivities of *E. coli* DNA-repair mutants transformed with the *katG* constructs

The transformants were tested for their ability to survive exposure to mitomycin C (6 µg ml⁻¹) for various time periods. Representative survival curves are shown in Fig. 4(c, d) and the results are summarized in Table 2. The parent *E. coli* strains MC1060 and AB1157 showed <10% killing at the exposure times used. The DK1 cells containing pSMKA, pSMK5' and pSMK3' survived mitomycin C treatment better than those harbouring vector alone (9-, 23-, and 25-fold increases in survival, respectively, relative to the control). In *E. coli* AB1886, all the *katG* constructs conferred increased resistance to mitomycin C (Fig. 4d, Table 2). In the other excision-repair mutants (AB1885 and AB1884) carrying the *katG* constructs, increased resistance to mitomycin C was also observed, but to a lesser extent (Table 2).

Hydrogen peroxide sensitivities of *E. coli* DNA-repair mutants transformed with the *katG* constructs

The transformants were tested for their ability to survive exposure to hydrogen peroxide (1.6 mg ml⁻¹) for various time periods. *E. coli* DK1 cells carrying pSMK3' appeared to be as sensitive to the oxidizing agent as those harbouring the vector alone, while the presence of pSMK5' and pSMKA increased the relative survival of the cells by 20- to 31-fold respectively, after 12 min exposure (Fig. 4e, Table 2). In the *E. coli* excision-repair mutants, no significant increase in resistance to hydrogen peroxide was conferred by the presence of any of the constructs (Fig. 4f, Table 2).

UV sensitivities of *katG* mutants of *M. tuberculosis* and *M. intracellulare*

The UV sensitivities of a *katG* mutant of *M. intracellulare* and its parent strain, *M. intracellulare* 1403, and of four *M. tuberculosis* clinical isolates, *M. tuberculosis* 255, *M. tuberculosis* 463, *M. tuberculosis* 519 and *M. tuberculosis* 34, were examined. *M. tuberculosis* 255 has no detectable mutations in the *katG* gene, *M. tuberculosis* 463 has mutations in codons 463 and 315, and *M. tuberculosis* 519 has a mutation in codon 315. These mutations map in the 5' region of the *katG* gene as defined in the constructs used in this study. The mutation in codon 315 results in a 50% reduction in enzyme activity (Rouse *et al.*, 1996), while the mutation in codon 463 has no significant effect on activity (Rouse *et al.*, 1996; Johnsson *et al.*, 1997). *M. tuberculosis* 34 has a complete deletion of the *katG* gene. The *katG* and *katG*⁺ *M. intracellulare* strains exhibited no differences in their sensitivity to UV (Fig. 6a). *M. tuberculosis* cells containing no detectable mutations in *katG* (isolate 255) exhibited a similar sensitivity to cells containing a

Fig. 6. UV survival curves of mycobacterial *katG* mutants. The curves represent the mean of three experiments; error bars represent the standard error of the mean. (a) *M. intracellulare* 1403 parent (●) and *katG* mutant strain (○). (b) Clinical isolates: ■, *M. tuberculosis* 463; □, *M. tuberculosis* 34; ○, *M. tuberculosis* 519; ●, *M. tuberculosis* 255.

mutation in codons 463 and 315 of *katG* (Fig. 6b). These strains exhibited a 36-fold reduction in survival at an UV dose of 60 $J m^{-2}$, relative to strains 519 and 34. They exhibited comparable survival to the latter strains at 120 $J m^{-2}$. It is important to note that the *M. tuberculosis* mutant strains are not isogenic.

DISCUSSION

Preliminary screening of an *M. tuberculosis* genomic DNA library on EMS suggested that the *katG* gene may be involved in DNA repair. Due to the lack of available *M. tuberculosis* repair mutants, we performed a heterologous study in well-characterized DNA-repair mutants of *E. coli*. These experiments showed that the *M. tuberculosis* KatG was able to partially complement *recA* mutant cells which had been exposed to UV light, hydrogen peroxide or mitomycin C. In cells mutant in *uvrA*, *uvrB* or *uvrC*, the increased resistance to damaging agents was only seen in response to mitomycin C exposure.

It is interesting that all three *katG* constructs showed similar effects in the *E. coli recA* mutants in response to UV and mitomycin C damage. If the 'protective activity' were encoded in the 5' portion of the gene, one would expect only pSMKA and pSMK5' to confer resistance, and that pSMK3' would have no effect. This is not the case, suggesting that the domains of the protein function independently, although the 3' domain is less effective. It also demonstrates that the protection is not due to the catalase or peroxidase activities of the protein, since neither domain is active on its own. However, the 20–30-fold increase in resistance to hydrogen peroxide treatment may reflect a contribution by these activities, which are absent in the 3' construct. Possibly the 5' portion of the KatG protein retains the ability to bind to hydrogen peroxide, thus reducing its ability to cause damage. As mentioned in Results, the two larger MBP–KatG fusion proteins appeared predominantly as cleavage products. From the size of these products, the cleavage site is predicted to lie approximately 75–95 codons from the C-terminus of the MBP–KatG5' fusion protein. It is possible that high amounts of full-length KatG may be toxic to the cells, thus promoting cleavage. It is also possible that the protective effects observed in these experiments are due predominantly to the cleavage products. This would explain why cells harbouring pSMKA and pSMK5' exhibit similar levels of resistance to the various DNA-damaging agents.

The results reported here suggest a novel, as yet undefined, activity of the *M. tuberculosis* enzyme. A possible explanation for the activity being encoded in both domains is the putative duplication of the *katG* gene. The N- and C-terminal sequences are similar, and it has been suggested that the C-terminal region arose from a gene-duplication event (Welinder, 1991, 1992). Another possible explanation is that the effect of the protein on the sensitivity of cells to UV is due to chelating of the intracellular iron, since both the 5' and 3' portions of KatG contain contact sites for haem. It was found that the presence of both domains did not have an additive effect, since mutants harbouring pSMKA generally exhibited similar resistance to those harbouring pSMK5'. However, since all the constructs were present in the cells on multicopy plasmids with gene expression from a strong promoter, the additive effects of both domains may not have been revealed.

Why would *M. tuberculosis* have evolved an additional KatG activity involved in DNA repair? *M. tuberculosis* has some unusual features with respect to DNA repair and oxidative stress mechanisms (Davis *et al.*, 1991, 1994; Deretic *et al.*, 1995; Sherman *et al.*, 1995; Dhandayuthapani *et al.*, 1996), and it is possible that it has evolved this activity in order to compensate for these differences. A review of the literature on the *E. coli katG* gene, however, suggests that this feature may not be unique to the *M. tuberculosis* KatG. A number of links have been observed between the presence or overexpression of KatG in *E. coli* cells, and both their resistance to, and spontaneous mutation rates from, various DNA-damaging agents (Farr *et al.*, 1988; Greenberg &

Demple, 1988; Hoerter *et al.*, 1989; Leven *et al.*, 1990). These observations were all attributed to the removal of reactive oxygen species by the catalase/peroxidase activity of KatG; however, oxygen radicals contribute only partly to the lethal effects of many of these agents. In the light of the results presented in this study on the *M. tuberculosis katG* gene, the above explanations may need to be reassessed.

The possibility of the *E. coli* KatG playing a role in DNA repair raises the question of its role in the experiments described in this paper. In the DNA-repair mutants of *E. coli* used, the resident *katG* gene is unaltered. The gene product is, therefore, probably produced at normal levels, and may have its own effect on the survival of cells after exposure to the various DNA-damaging agents. This effect will, however, be present in strains carrying the vector control as well as the fusion constructs. Thus what is being measured in these experiments is the additive effect of the *M. tuberculosis* KatG, which is present in excess to the endogenous KatG on induction.

Overexpression of the foreign proteins could be responsible for the effects observed in these experiments. Kanemori *et al.* (1994), for example, have demonstrated that the production of abnormal proteins in *E. coli* results in enhanced synthesis of heat-shock proteins due to an increase in the stabilization of σ^{32} . In addition, Tsui *et al.* (1996) demonstrated an induction of DNA-repair pathways upon induction of the heat-shock response. Thus it is possible that the modification of DNA repair seen in *E. coli* on expression of the *M. tuberculosis katG* gene could be the result of non-specific effects due to the high-level expression of a heterologous protein. However, overexpression of the MBP-LacZ fusion and the fusions produced by the pSMK3'a, pSMK3'b, pSMK3'opp and pSMKd constructs did not result in increased resistance to UV light. Only fusion proteins containing the *M. tuberculosis* KatG showed this effect. In addition, protection of the cells was strain-specific. Thus it is unlikely that the effects observed in these experiments are due to the non-specific effects described above.

In this study, no differences were observed in the far-UV sensitivities of *katG* and *katG*⁺ strains of *M. intracellulare*. The mutant strain was constructed by allelic exchange, and is, therefore, isogenic with the parent strain, except with respect to *katG*. These results suggest that in repair-competent hosts (i.e. cells in which no DNA-repair genes have been mutated) the *katG* gene does not play a demonstrable role in repair of damage caused by UV irradiation. The *M. tuberculosis* clinical isolates available for this study are, however, not isogenic, and the effect of mutations in *katG* on sensitivity to UV cannot be compared directly. *M. tuberculosis* strain 34, for example, has recently been shown to overexpress AhpC due to mutations in the promoter region of the *ahpC* gene (Heym *et al.*, 1997). The increased expression of this protein may compensate for the lack of KatG in this strain. The mycobacterial UV experiments were all performed using

repair-competent hosts that exhibit a high natural resistance to UV irradiation. Any effects of *katG* on the UV sensitivities of these strains may, therefore, be masked. Investigation of the role of *katG* in DNA repair in the mycobacteria will, therefore, require complementation studies with the gene in isogenic repair-deficient mycobacterial hosts. The recent advances in the construction of defined mutants of both the rapidly growing mycobacteria and organisms of the *M. tuberculosis* complex should facilitate this kind of study in the future.

ACKNOWLEDGEMENTS

The University of Cape Town and the Medical Research Council are acknowledged for grants. This work was performed as part of the Glaxo Wellcome Action TB initiative. We would like to thank Drs S. Cole, B. Marklund and T. Victor for mycobacterial strains.

REFERENCES

- Abratt, V. R., Jones, D. T. & Woods, D. R. (1985). Isolation and physiological characterization of mitomycin C-sensitive/UV-sensitive mutants in *Bacteroides fragilis*. *J Gen Microbiol* **131**, 2479–2483.
- Altamirano, M., Marostenmaki, J., Wong, A., FitzGerald, M., Black, W. A. & Smith, J. A. (1994). Mutations in the catalase-peroxidase gene from isoniazid-resistant *Mycobacterium tuberculosis* isolates. *J Infect Dis* **169**, 1162–1165.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Chung, C. T. & Miller, R. H. (1988). A rapid and convenient method for the preparation and storage of competent bacterial cells. *Nucleic Acids Res* **16**, 3580.
- Clare, D. A., Duong, M. N., Darr, D., Archibald, F. & Fridovich, I. (1984). Effects of molecular oxygen on detection of superoxide radical with nitroblue tetrazolium and on activity stains for catalase. *Anal Biochem* **140**, 532–537.
- Crane, D., Holmes, R. & Masters, C. (1982). Proteolytic modification of mouse liver catalase. *Biochem Biophys Res Commun* **104**, 1567–1572.
- Davis, E. O., Sedgwick, S. G. & Colston, M. J. (1991). Novel structure of the *recA* locus of *Mycobacterium tuberculosis* implies processing of the gene product. *J Bacteriol* **173**, 5653–5662.
- Davis, E. O., Thangaraj, H. S., Brooks, P. C. & Colston, M. J. (1994). Evidence of selection for protein introns in the RecAs of pathogenic mycobacteria. *EMBO J* **13**, 699–703.
- Demple, B., Halbrook, J. & Linn, S. (1983). *Escherichia coli xth* mutants are hypersensitive to hydrogen peroxide. *J Bacteriol* **153**, 1079–1082.
- Deretic, V., Philipp, W., Dhandayuthapani, S., Mudd, M. H., Curcic, R., Garbe, T., Heym, B., Via, L. E. & Cole, S. T. (1995). *Mycobacterium tuberculosis* is a natural mutant with an inactivated oxidative-stress regulatory gene: implications for sensitivity to isoniazid. *Mol Microbiol* **17**, 889–900.
- Dhandayuthapani, S., Zhang, Y., Mudd, M. H. & Deretic, V. (1996). Oxidative stress response and its role in sensitivity to isoniazid in mycobacteria: characterization and inducibility of *ahpC* by peroxides in *Mycobacterium smegmatis* and lack of expression in *M. aurum* and *M. tuberculosis*. *J Bacteriol* **178**, 3641–3649.
- Durbach, S. I., Andersen, S. J. & Mizrahi, V. (1997). SOS induction

- in mycobacteria: analysis of the DNA-binding activity of a LexA-like repressor and its role in DNA damage induction of the *recA* gene from *Mycobacterium smegmatis*. *Mol Microbiol* **26**, 643–653.
- Farr, S. B., Touati, D. & Kogoma, T. (1988). Effects of oxygen stress on membrane functions in *Escherichia coli*: role of HPI catalase. *J Bacteriol* **170**, 1837–1842.
- Friedberg, E. C., Walker, G. C. & Siede, W. (1995). *DNA Repair and Mutagenesis*, Washington, DC: American Society for Microbiology.
- Greenberg, J. T. & Demple, B. (1988). Overproduction of peroxide-scavenging enzymes in *Escherichia coli* suppresses spontaneous mutagenesis and sensitivity to redox-cycling agents in *oxyR*⁻ mutants. *EMBO J* **7**, 2611–2617.
- Henikoff, S. (1987). Unidirectional digestion with exonuclease III in DNA sequence analysis. *Methods Enzymol* **155**, 156–165.
- Heym, B., Zhang, Y., Poulet, S., Young, D. & Cole, S. T. (1993). Characterization of the *katG* gene encoding a catalase-peroxidase required for the isoniazid susceptibility of *Mycobacterium tuberculosis*. *J Bacteriol* **175**, 4255–4259.
- 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 short-course chemotherapy of tuberculosis: a molecular study. *Lancet* **344**, 293–298.
- Heym, B., Stavropoulos, E., Honore, N., Domenech, P., Saint-Joanis, B., Wilson, T. M., Collins, D. M., Colston, M. J. & Cole, S. T. (1997). Effects of overexpression of the alkyl hydroperoxide reductase AhpC on the virulence and isoniazid resistance of *Mycobacterium tuberculosis*. *Infect Immun* **65**, 1395–1401.
- Hoerter, J., Eisenstark, A. & Touati, D. (1989). Mutations by near-ultraviolet radiation in *Escherichia coli* strains lacking superoxide dismutase. *Mutat Res* **215**, 161–165.
- Howard-Flanders, P., Boyce, R. P. & Theriot, L. (1966). Three loci in *Escherichia coli* K-12 that control the excision of pyrimidine dimers and certain other mutagen products from DNA. *Genetics* **53**, 1119–1136.
- Jackett, P. S., Aber, V. R. & Lowrie, D. B. (1978). Virulence and resistance to superoxide, low pH and hydrogen peroxide among strains of *Mycobacterium tuberculosis*. *J Gen Microbiol* **104**, 37–45.
- Johnsson, K., Froland, W. A. & Schultz, P. G. (1997). Overexpression, purification, and characterization of the catalase-peroxidase KatG from *Mycobacterium tuberculosis*. *J Biol Chem* **272**, 2834–2840.
- Jones, D. T. & Woods, D. R. (1981). Effect of oxygen on liquid holding recovery of *Bacteroides fragilis*. *J Bacteriol* **145**, 1–7.
- Kanemori, M., Mori, H. & Yura, T. (1994). Induction of heat shock proteins by abnormal proteins results from stabilization and not increased synthesis of sigma 32 in *Escherichia coli*. *J Bacteriol* **176**, 5648–5653.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Leven, S., Heimberger, A. & Eisenstark, A. (1990). Catalase HPI influences membrane permeability in *Escherichia coli* following near-UV stress. *Biochem Biophys Res Commun* **171**, 1224–1228.
- Meissner, P. S., Sisk, W. P. & Berman, M. L. (1987). Bacteriophage λ cloning system for the construction of directional cDNA libraries. *Proc Natl Acad Sci USA* **84**, 4171–4175.
- Middlebrook, G. (1954). Isoniazid resistance and catalase activity of tubercle bacilli: a preliminary report. *Am Rev Tuberc Pulm Dis* **69**, 471–472.
- Middlebrook, G. & Cohn, M. L. (1953). Some observations on the pathogenicity of isoniazid-resistant variants of tubercle bacilli. *Science* **118**, 297–299.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mitchison, D. A., Wallace, J. G., Bhatia, A. L., Selkon, J. B., Subbaiah, J. & Lancaster, M. C. (1960). A comparison of the virulence in guinea pigs of South India and British tubercle bacilli. *Tubercle* **41**, 1–22.
- Mizrahi, V. & Andersen, S. J. (1998). DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? *Mol Microbiol* **29**, 1331–1339.
- Movahedzadeh, F., Colston, M. J. & Davis, E. O. (1997a). Characterization of *Mycobacterium tuberculosis* LexA: recognition of a Cheo (*Bacillus*-type SOS) box. *Microbiology* **143**, 929–936.
- Movahedzadeh, F., Colston, M. J. & Davis, E. O. (1997b). Determination of DNA sequences required for regulated *Mycobacterium tuberculosis* RecA expression in response DNA-damaging agents suggests that two modes of regulation exist. *J Bacteriol* **179**, 3509–3518.
- Mulvey, M. R., Sorby, P. A., Triggs-Raine, B. L. & Loewen, P. C. (1988). Cloning and physical characterization of *katE* and *katF* required for catalase HPII expression in *Escherichia coli*. *Gene* **73**, 337–345.
- Nair, S. & Steyn, L. M. (1991). Cloning and expression in *Escherichia coli* of a *recA* homologue from *Mycobacterium tuberculosis*. *J Gen Microbiol* **137**, 2409–2414.
- Papavinasasundaram, K. G., Movahedzadeh, F., Keer, J. T., Stoker, N. G., Colston, M. J. & Davis, E. O. (1997). *Mycobacterial recA* is cotranscribed with a potential regulatory gene called *recX*. *Mol Microbiol* **24**, 141–153.
- Robbi, M. & Lazarow, P. B. (1978). Synthesis of catalase in two cell-free protein-synthesizing systems and in rat liver. *Proc Natl Acad Sci USA* **75**, 4344–4348.
- Rouse, D. A. & Morris, S. L. (1995). Molecular mechanisms of isoniazid resistance in *Mycobacterium tuberculosis* and *Mycobacterium bovis*. *Infect Immun* **63**, 1427–1433.
- Rouse, D. A., DeVito, J. A., Li, Z., Byer, H. & Morris, S. L. (1996). Site-directed mutagenesis of the *katG* gene of *Mycobacterium tuberculosis*: effects on catalase-peroxidase activities and isoniazid resistance. *Mol Microbiol* **22**, 583–592.
- Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* **239**, 487–491.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Sherman, D. R., Sabo, P. J., Hickey, M. J., Arain, T. M., Mahairas, G. G., Yuan, Y., Barry, C. E., III & Stover, C. K. (1995). Disparate responses to oxidative stress in saprophytic and pathogenic mycobacteria. *Proc Natl Acad Sci USA* **92**, 6625–6629.
- Stoeckle, M. Y., Guan, L., Riegler, N., Weitzman, I., Kreiswirth, B., Kornblum, J., Laraque, F. & Riley, L. W. (1993). Catalase-peroxidase gene sequences in isoniazid-sensitive and -resistant strains of *Mycobacterium tuberculosis* from New York City. *J Infect Dis* **168**, 1063–1065.
- Tsui, H. C., Feng, G. & Winkler, M. E. (1996). Transcription of the *mutL* repair, *miaA* tRNA modification, *hfq* pleiotropic regulator,

and *hflA* region protease genes of *Escherichia coli* K-12 from clustered E sigma(32)-specific promoters during heat shock. *J Bacteriol* **178**, 5719–5731.

Wayne, L. G. & Diaz, G. A. (1986). A double staining method for differentiating between two classes of mycobacterial catalase in polyacrylamide electrophoresis gels. *Anal Biochem* **157**, 89–92.

Welinder, K. G. (1991). Bacterial catalase-peroxidases are gene duplicated members of the plant peroxidase superfamily. *Biochim Biophys Acta* **1080**, 215–220.

Welinder, K. G. (1992). Superfamily of plant, fungal and bacterial peroxidases. *Curr Opin Struct Biol* **2**, 388–393.

Willis, D. K., Uhlin, B. E., Amini, K. S. & Clark, A. J. (1981). Physical mapping of the *srl recA* region of *Escherichia coli*: analysis of Tn10 generated insertions and deletions. *Mol Gen Genet* **183**, 497–504.

Wilson, T. M., de Lisle, G. W. & Collins, D. M. (1995). Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. *Mol Microbiol* **15**, 1009–1015.

Winder, F. G. (1960). Catalase and peroxidase in mycobacteria: possible relationship to the mode of action of isoniazid. *Am Rev Resp Dis* **81**, 68–78.

Zappe, H., Jones, D. T. & Woods, D. R. (1986). Cloning and expression of *Clostridium acetobutylicum* endoglucanase, cellobiase and amino acid biosynthesis genes in *Escherichia coli*. *J Gen Microbiol* **132**, 1367–1372.

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.

Received 8 February 1999; accepted 13 April 1999.