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Identification and characterization of a diamide sensitive mutant of *Mycobacterium smegmatis*

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Abstract

A mutant, T7, highly sensitive to oxidative stress as caused by diamide was isolated from a *Mycobacterium smegmatis* mc^{2}155 transposon mutant library. While wild-type *M. smegmatis* is able to grow well on solid media supplemented with 10 mM diamide, T7 is only able to grow on solid media containing up to 1 mM diamide. This mutant is also sensitive to other thiol modifying agents such as iodoacetamide and chlorodinitrobenzene. By sequencing the genomic DNA flanking the transposon, T7 was found to be mutated in the region upstream of the homolog of *M. tuberculosis* Rv0274 open reading frame. Sequence analysis revealed that Rv0274 is a member of a superfamily of metalloenzymes comprising enzymes such as extradiol dioxygenases, glyoxalases, and fosfomycin resistant glutathione transferases. Cloning and epichromosomal expression of *M. tuberculosis* Rv0274 in the mutant resulted in complementation of the sensitivity to diamide.

Keywords: Disulfide; Mycothiol; Transposon mutant; VOC superfamily; Detoxification

1. Introduction

Mycobacterium tuberculosis, a facultative intracellular pathogen, is the causative agent of tuberculosis (TB). Over one-third of the world's population is infected with TB and approximately 2 million people die from the disease yearly [1]. The fact that *M. tuberculosis* is able to enter mononuclear phagocytic cells and survive and replicate within the phagosomes is crucial to its pathogenesis. Oxidative stress response and protection against reactive oxygen intermediates and reactive nitrogen intermediates released during the oxidative burst in the phagosome have been implicated in the intracellular survival and persistence of *M. tuberculosis* in human macrophages [2].

In eukaryotes and Gram-negative prokaryotes, glutathione, a low molecular mass thiol, plays an important role in defending the organisms against oxidative stress and environmental toxins. Glutathione dependent enzymes such as glutathione peroxidases and glutathione *S*-transferases protect the cell against oxidants and electrophiles [3]. Actinomycetes like mycobacteria do not produce glutathione but instead synthesize mycothiol, a thiol comprised of *N*-acetyl-L-cysteine linked to a pseudodisaccharide, D-glucosamine and *myo*-inositol [4]. Since mycobacteria lack glutathione, mycothiol serves analogous functions to glutathione in these organisms including protection against oxidants. For instance, *Mycobacterium smegmatis*, a fast-growing non-pathogenic relative of *M. tuberculosis*, is able to withstand up to 12 mM hydrogen peroxide without loss of viability whereas a chemically induced mutant and transposon mutants lacking mycothiol are killed by hydrogen peroxide concentration of 1 mM [5,6]. *M. smegmatis* is also remarkable in its ability to withstand millimolar concentrations of toxins such as monobromobimane, an alkylating agent that is lethal in much smaller amounts to cultured mammalian cells [5]. Mycothiol may, thus, play an important role in protecting *M. tuberculosis* against hostile environments such as that of the macrophages.

Diamide has been used extensively as a chemical probe to study the effect of changes in the oxidation state of thiols [7]. It readily penetrates the cell and causes oxidative

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Abbreviations: TB, tuberculosis; OADC, oleic acid, albumin, dextrose, catalase supplement; LB, Lennox L broth; CDNB, 1-chloro-2,4-dinitrobenzene; PCR, polymerase chain reaction; VOC vicinal-oxygen-chelate; OD, optical density

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stress by specifically oxidizing intracellular thiols as illustrated by the following reaction: $(CH_3)_2NCO–N=N–CON(CH_3)_2+2RSH \rightarrow (CH_3)_2NCO–NHHN–CON(CH_3)_2+RSSH$. Diamide has been used to obtain *Escherichia coli* mutants defective in glutathione biosynthesis [8]. In addition, diamide sensitivity is also associated with glutathione reductase mutants [9] and mutations in transcriptional factors that regulate response to oxidative stress in *Streptomyces coelicolor* and *Saccharomyces cerevisiae* [10,11]. Recently, Raman et al. [12] reported that a 'knockout' mutant of sigma factor, SigH, which regulates transcription in response to thiol oxidation in *M. tuberculosis*, is sensitive to diamide. This mutant is also sensitive to a variety of reagents including hydrogen peroxide and redox cycling agents that produce superoxide radicals. Moreover, mutants lacking mycothiol are extremely sensitive to diamide [5,6]. Incidentally, mycothiol deficient mutants are also sensitive to other oxidative stresses such as hydrogen peroxide and redox cycling agents.

To identify other mycobacterial genes associated with protection against disulfide damaging oxidative stress, we constructed a transposon mutant library in *M. smegmatis* and screened this library for diamide sensitive mutants. In this report, we describe one of the mutants isolated and show that the genetic lesion is upstream of the *M. smegmatis* homolog of the Rv0274 *M. tuberculosis* gene. Unlike previously reported diamide sensitive mutants, the mutant phenotype is restricted to disulfide oxidizing stress.

2. Materials and methods

2.1. Bacterial strains and culture conditions

E. coli DH5α was used as the host stain for cloning experiments. *M. smegmatis* mc^{2}155 was the parent strain from which transposon mutants were constructed. *M. smegmatis* was grown in Middlebrook 7H9 broth (Difco) with 0.05% Tween and supplemented with either Middlebrook oleic acid, albumin, dextrose, catalase supplement (OADC) or 1% glucose. *M. smegmatis* was also grown on Middlebrook 7H10 solid medium (Difco) supplemented with OADC or 1% glucose. Cultures were also grown on Lennox L (LB) broth and LB solid medium. Ampicillin (100 μg ml^{-1}), kanamycin (25 μg ml^{-1}), and hygromycin (100 μg ml^{-1} for *E. coli* and 50 μg ml^{-1} for *M. smegmatis*). Complements of mutants harboring the recombinant PALACE vector were grown on Middlebrook 7H10 solid medium supplemented with OADC and 2% acetamide for induction of the cloned gene. Antibiotics were added as appropriate.

2.2. Molecular biology techniques

Genomic DNA was isolated from *M. smegmatis* cultures according to Larsen [13]. Standard recombinant DNA techniques such as restriction digestions, ligations, and transformations were carried out as described by Sambrook et al. [14]. The strains, plasmids and oligonucleotides used in this study are described in Table 1.

2.3. Southern blots

A DIG labeling system was used to prepare the probes according to the manufacturer's instructions (Boehringer Mannheim). To prepare the probe for the transposon, the kan5′ and kan3′ primers (Table 1) were used to amplify the kanamycin cassette from the genomic DNA of T7 transposon mutant. To prepare the probe for Rv0274, the sequence corresponding to Rv0274 in the *M. smegmatis* incomplete sequence (http://www.tigr.org) was used to prepare primers for polymerase chain reaction (PCR) amplification. 0274smeg5′ and 0274smeg3′ (Table 1) were used to amplify the mc^{2}155 genomic DNA for the production of the probe.

2.4. Construction and screening of transposon library

The transposon library (EZ::TN ⟨kan-2⟩Tnp transposase and Tn903 kanamycin resistance marker) was constructed according to the manufacturer's instructions, Epicenter Technologies. Briefly, 1 μl of the EZ::TN transposome was electroporated into electrocompetent *M. smegmatis* mc^{2}155 cells that had been prepared according to Snapper et al. [15]. The cells were then plated on Middlebrook 7H10 solid medium supplemented with OADC and 25 μg ml^{-1} kanamycin and allowed to grow for 2–3 days at 37°C. Four hundred colonies were picked and grown on ELISA plates and glycerol stocks were made of the mutants. The mutants were then screened on Middlebrook 7H10 solid medium supplemented with 25 μg ml^{-1} kanamycin and 10 mM diamide. Any mutant that failed to grow on the diamide plates was tested further for diamide sensitivity.

2.5. Identification of the site of insertion of the transposon

A 5 μg amount of genomic DNA from T7 mutant was digested with restriction enzyme *Pst*I and self-ligated under dilute conditions overnight at 14°C. PCR amplification of the ligation mixture was performed using reverse primers provided in the EZ::TN ⟨KAN-2⟩Tnp transposome kit, Kan-2Fp-1 and Kan-2Rp-1 5′ (Table 1). The PCR product was gel purified and then sequenced using the primers Kan-2Fp-1 and Kan-2Rp-1. The sequence obtained was searched using the BLAST program [16] against TIGR microbial databases and NCBI nucleotide and protein databases through the Entrez browser at http://www.ncbi.nlm.nlh.gov/Entrez. Sequence data for *M. tuberculosis*, *Mycobacterium leprae* and *Mycobacterium bovis* were obtained from The Wellcome Sanger Institute website at http://www.sanger.ac.uk/Projects/M_tuberculo-

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

Strain, plasmid, oligonucleotide	Characteristics[a]	Source or reference
Strains		
M. tuberculosis	H37Rv	NCTC7416
M. smegmatis	mc^{2}155, parent strain	W.R. Jacobs
E. coli DH5α	*F*$^-$ *rec*A1 *hsd*R17 *thi*-1 *gyr*A96 *sup*E44 *end*A1 *rel*A1 *rec*A1 *deo*R Δ(*lac*ZYa-*arg*F)U169(ϕ80 *lac*Z ΔM15)	
T7	*M. smegmatis* mutant in Rv0274	This study
T7::palace0274	T7 complemented with *M. tuberculosis* Rv0274	This study
T7::pSodit0274	T7 complemented with *M. tuberculosis* Rv0274	This study
Plasmids		
PALACE	Hyg^r	K. De Smet
pSODIT-2	Hyg^r	K. De Smet
pCR2.1	TA cloning vector, Amp^r	Invitrogen
pMR0274A	PCR amplified Rv0274 cloned into pCR2.1	This study
pMR0274B	*Bam*HI/*Eco*RV digested Rv0274 ligated into pSODIT-2	This study
Oligonucleotides		
Kan5′	5′-CTC AAA ATC TCT GAT GTT ACA TTG C-3′	This study
Kan3′	5′-GGT TGA TGA GAG CTT TGT TGT-3′	This study
0274smeg5′	5′-ATG ATC AGA CCC GAC AAC CCC-3′	This study
0274smeg3′	5′-CGG TCG GCC GCG GTC TTC-3′	This study
Kan-2-Fp-1	5′-ACC TAC AAA GCT CTC ATC AAC C-3′	Epicenter Technologies
Kan-2-Rp-1	5′-GCA ATG TAA CAT CAG AGA TTT TGA G-3′	Epicenter Technologies
pal0274f	5′-TTG GGA TCC ATG ATC AAG CCG CAC AA-3′	This study
pal0274r	5′-CAC ATC GAT CTA ACG ATC CGC AGC CA-3′	This study
P1	5′-TGG TAG GCC GCC ATC AAC GA-3′	This study
P2	5′-AGC TGC GAC ACG CTG AT-3′	This study
P3	5′-ACA CCT TCC GCG AGA ACG CC-3′	This study

[a]Amp^r, ampicillin resistance; Hyg^r, hygromycin resistance; SOD, superoxide dismutase.

sis/. Preliminary sequence data of *M. smegmatis* and *Mycobacterium avium* were obtained from The Institute for Genomic Research website at http://www.tigr.org. Alignments were performed through the CLUSTAL W1.8 program at http://searchlauncher.bcm.tmc.edu and through the boxshade program at http://www.ch.embnet.org/software/BOX_form.html. To ascertain whether the site of insertion was in the open reading frame (ORF) obtained from the BLAST search, PCR amplifications were performed with sets of primers: 0274smeg5′ and 0274smeg3′; P1 and P3; and P2 and 0274smeg3′ (Table 1).

2.6. Toxin and antibiotic sensitivity assays

Disk assays were performed to determine sensitivity of the mutant to various stresses. Briefly, cells were grown to mid-exponential phase and a lawn of cells was plated onto LB plates (mc^{2}155) or LB plates supplemented with kanamycin (T7 mutant). Various amounts of compounds to be tested were added to paper filter disks in a 10-μl volume and allowed to dry. The disks were placed onto the lawn of cells and the plates were incubated for 3 days. The following redox cycling agents were tested: nitrofurantoin (0.1–1.0 μmol), paraquat, menadione (0.1–0.5 μmol), and plumbagin (0.001–0.01 μmol). Cumene hydrogen peroxide (0.01–5.0 μmol), an organic peroxide, was also tested. Sensitivity to methylglyoxal (0.1–1.0 μmol), a substrate for the enzyme glyoxalase, and catechol (0.1–25 μmol), a substrate for an extradiol dioxygenase, was also tested. Sensitivity to the frontline drug against TB, isoniazid (0.5–50 μg), was tested.

Initially, diamide, dichloronitrobenzene (CDNB), and iodoacetamide were also tested using disk assays. To confirm the differing sensitivity of the mutant from the parent seen in the disk assays and to determine the sensitivity of the complement of the mutant to diamide, solid media supplemented with the appropriate antibiotics and differing concentrations of diamide (1, 2.5, 5.0, 7.5, 10, 12.5, 15, 30 mM), CDNB (0.005, 0.01, 0.025, 0.05, 0.075 mM), and iodoacetamide (0.005, 0.01, 0.015, 0.020, 0.025, 0.030, 0.035, 0.05 mM) were poured and bacteria were streaked onto these plates. Chemicals for the disk sensitivity assay were obtained from Sigma. All assays were done in triplicate in at least three separate experiments.

2.7. Liquid growth assays

Stock bacterial cultures of T7 and mc^{2}155 were diluted to 0.05–0.1 OD_{600nm} with Middlebrook 7H9 medium supplemented with OADC and appropriate antibiotics. The optical density (OD) was measured at various time intervals with a Beckman DU 640 spectrophotometer using a

cuvette with a pathlength of 1 cm. To determine the effect of CDNB and iodoacetamide on growth, either 0.01 mM CDNB or 0.01 mM iodoacetamide was added to the liquid growth medium. The cultures were incubated at 37°C in the dark and samples of the cultures were taken at various time points and optical density at 600 nm was measured.

To determine the effect of diamide on growth, various concentrations of diamide were added to the liquid cultures and the cultures were also incubated at 37°C in the dark. Diamide in its oxidized form when dissolved in aqueous solution has an extinction coefficient of 3000 M^{-1} cm^{-1} at 296 nm and when it is reduced, the species formed does not absorb in the ultraviolet range allowing diamide to be monitored by measuring the absorbance of the solution at 296 nm. Samples of the cultures were taken at various time points and the absorbance at 295 nm was measured along with the optical density at 600 nm. The experiments were repeated at least three times.

2.8. Complementation of T7

The *M. tuberculosis* Rv0274 gene was amplified from genomic DNA using the following primers: pal0274f and pal0274r (Table 1). The forward and reverse primers had a *Bam*HI site and *Cla*I site engineered into the primers respectively for cloning into PALACE *M. smegmatis*/*E. coli* shuttle vector generously provided by Dr. K. De Smet [17]. The *M. tuberculosis* Rv0274 gene was PCR amplified and the PCR product was cloned into pCR2.1 vector. For cloning Rv0274 into PALACE, both pCR2.1 vector containing Rv0274 and empty PALACE vector were digested with *Bam*HI and *Cla*I and ligated overnight. In addition, the pCR2.1 vector containing Rv0274 was also digested with *Bam*HI and *Eco*RV for ligation to *Bam*HI/*Eco*RV digested pSODIT-2 vector also provided by Dr. K. De Smet. The ligation mixtures were transformed into *E. coli* DH5α cells and plated onto LB agar plates containing 100 μg ml^{-1} hygromycin. The transformants were then screened by restriction digest analysis of the construct DNA, and once confirmed, the constructs was electroporated into T7 electrocompetent cells [15] and plated onto LB agar plates supplemented with 50 μg ml^{-1} hygromycin and 25 μg ml^{-1} kanamycin. Transformants were screened by SDS–PAGE analysis of the protein extract.

3. Results

3.1. Production and screening of a partial M. smegmatis transposon mutant library

A partial *M. smegmatis* transposon mutant library consisting of 400 individual mutants was created using EZ::TN transposome kit (Epicenter Technologies). The mutant colonies were picked, sorted and cultured in ELISA plates. To screen for diamide sensitive mutants, the mutants were replica plated on LB plates supplemented with 25 μg ml^{-1} kanamycin and LB plates supplemented with 25 μg ml^{-1} kanamycin and 10 mM diamide. Two mutants failed to grow on diamide containing plates. Here, we report the result of studies for one of these mutants, T7. The second mutant is disrupted in a hypothetical gene involved in fatty acid metabolism. To confirm that T7 was indeed a transposon mutant, Southern hybridization with the transposon was performed (Fig. 1A). In lanes 1 and 4, which contain mc^2155 DNA digested with *Pst*I and *Bam*HI, respectively, there is no hybridization with the transposon probe while in lanes 2 and 3, which contain T7 DNA, digested with *Pst*I and *Bam*HI, respectively, there is hybridization. In lane 2, a band of 2.25 kb is present and in lane 3, a band of 2.75 kb is present indicating the presence of a single transposon in the T7 mutant.

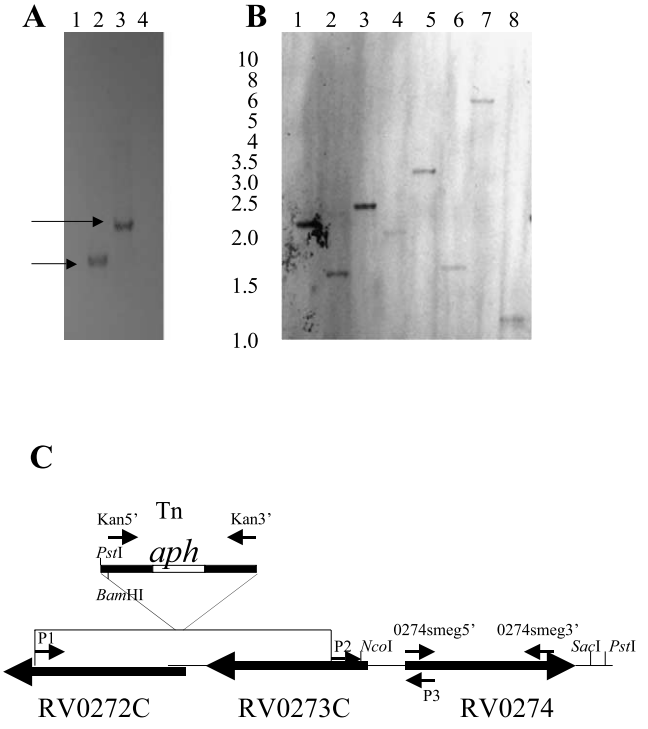

Fig. 1. Southern blot analysis of *M. smegmatis* mc^2155 and T7. 10 μg of mc^2155 and T7 genomic DNA was loaded on each lane. A: Southern blot probed with kanamycin resistance gene for the presence of the transposon; Lanse 1 and 2, mc^2155 and T7 genomic DNA cut with *Pst*I respectively; lanes 3 and 4, T7 and mc^2155 genomic DNA cut with *Bam*HI, respectively. B: Southern blot probed with *M. smegmatis* Rv0274 homolog. Lanes1–4, T7 genomic DNA cut with *Pst*I, *Nco*I, *Bam*HI, and *Sac*I respectively; lanes 5–8, mc^2155 genomic DNA cut with *Pst*I, *Nco*I, *Bam*HI, and *Sac*I respectively. C: Schematic representation of the transposon site of insertion in T7. The site of transposon insertion is in the area between the sites for primers P1 and P2 as determined by PCR amplification of the T7 DNA.

3.2. T7 is disrupted in the upstream region of a homolog of M. tuberculosis Rv0274 ORF

The disrupted gene was identified by sequencing the

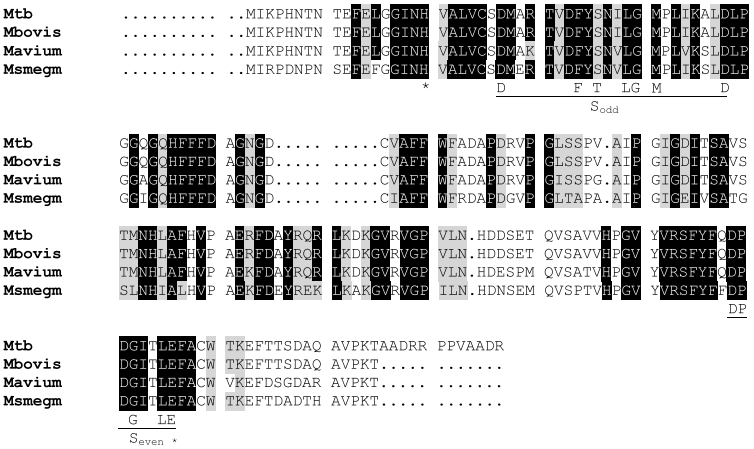

Fig. 2. Alignment of Rv0274 and its homologs in mycobacteria (Mtb, *M. tuberculosis*; Mbovis, *M. bovis*; Mavium, *M. avium*; Msmeg, *M. smegmatis*). The VOC superfamily signature sequences, S_{even} = (D,T)PXGX$_2$(L,V,I)(E,H) and S_{odd} = DX$_6$FXTX$_2$LG(F,M,L)X$_6$D in Rv0274 are shown below the aligned sequences and the location of the putative metal ligands is designated with an asterisk.

genomic DNA flanking the transposon. *M. tuberculosis* Rv0274 was identified through a BLAST search of the resultant sequence against the *M. tuberculosis* database at http://www.sanger.ac.uk. The sequence obtained was also BLAST searched against the incomplete *M. smegmatis* sequence. The sequence matched identically the Rv0274 homolog in *M. smegmatis*, indicating that the site of insertion was upstream of the Rv0274 homolog in *M. smegmatis*. Southern hybridization with *M. smegmatis* homolog of Rv0274 was performed (Fig. 1B). Genomic DNA from T7 and mc^{2}155 was digested with *Pst*I (lanes 1 and 5, respectively) and *Bam*HI (lanes 3 and 7), two enzymes for which there are restriction sites within the transposon and downstream of the *M. smegmatis* Rv0274 gene, *Nco*I (lanes 2 and 6), for which there is a restriction site upstream of the gene in Rv0273c and a restriction site downstream of Rv0274, and *Sac*I (lanes 4 and 8), for which there are restriction sites upstream and downstream of the gene. In Fig. 1B, it can be seen that the probe hybridizes with a smaller band in the T7 DNA in the *Pst*I and *Bam*HI lanes, the probe hybridizes with a band of similar size in the *Nco*I lane, and the probe hybridizes with a larger band in the T7 DNA in the *Sac*I lane.

Because Southern hybridization indicated that the site of transposon insertion was not in the *M. smegmatis* homolog of Rv0274, primers upstream of the region were designed (Table 1). PCR amplification with primers 0274smeg5′ and 0274smeg3′ and primers P2 and 0274smeg3′ of DNA from parent strain and T7 did not show a difference in the size of the band amplified while PCR amplification with primers P1 and P3 resulted in a band that was larger by 1.2 kb (the size of the transposon) in T7 DNA as compared to the parent DNA (data not shown). The PCR results indicated that the site of insertion was between sites for primers P1 and P2 (Fig. 1C).

3.3. Sequence analyses of Rv0274

In the *M. tuberculosis* genome sequence, Rv0272c is annotated as a hypothetical unknown protein, Rv0273c as a putative transcriptional regulatory protein and Rv0274 as a protein of unknown function having an extradiol ring cleavage signature near its C-terminus. Rv0274 is 579 bp long and encodes a protein of 193 amino acids with a molecular mass of 21.1 kDa. The p*I* for this protein is 5.54 and it is presumed to be soluble. In an attempt to assign a function to the protein encoded by ORF Rv0274, we tried to identify homologs of this protein. A BLAST search of GenBank as well as the data in the unfinished microbial genomes was performed. Not surprisingly, as seen in Fig. 2, the most conserved homologs of Rv0274 were found in other mycobacterial species indicating the presence of Rv0274 in all the mycobacterial sequences available today.

Unfortunately, most of the homologs with a significant score (above 25) identified by the BLAST search were of an unknown function. Nevertheless, our search revealed that the protein encoded by Rv0274 has a low degree of similarity to proteins such as extradiol dehydrogenase, fosfomycin resistance glutathione transferases, bleomycin resistance protein, methylmalonyl CoA epimerases, and glyoxalases from various species. These enzymes belong to a vicinal-oxygen-chelate (VOC) superfamily which has a characteristic βαββ fold. Members of this family catalyze divalent metal ion dependent reactions that require stabilization of oxyanion intermediates. Of all the known members of the VOC superfamily, only bleomycin resistance protein does not bind any divalent metal ion [18]. Analysis of the Rv0274 sequence indicates that Rv0274 contains the putative metal binding ligands, histidine and aspartic acid, that are present in the other members of this superfamily [19]. Moreover, Rv0274 contains the distinct signature sequences, S_{even}, and S_{odd}, characteristic of the

superfamily [20]. The only difference in the Rv0274 amino acid sequence from the VOC superfamily signature sequences is the replacement of threonine with functionally similar serine in the S_{odd} signature sequence (Fig. 2). This together with the above data led us to the conclusion that Rv0274 is a member of a unique mycobacterial family of proteins which has some similarity to the VOC superfamily.

3.4. T7 mutant is sensitive to diamide

Initially, T7 was identified as a mutant unable to grow on media containing 10 mM diamide. Disk assays to determine the extent of diamide sensitivity in this mutant showed clearing at 0.005 μmol while mc^{2}155 shows no clearing even at 0.01 μmol (data not shown). When mc^{2}155 and T7 were streaked on LB plates containing different concentrations of diamide, T7 was able to grow on LB plates containing 1 mM diamide or less while mc^{2}155 was able to grow as well on 10 mM diamide indicating that the mutant is at least 10 times more sensitive to diamide than mc^{2}155 (Table 2).

3.5. Diamide detoxification is time and dose dependent

As seen in Fig. 3A, the T7 growth rate is slightly less than the growth rate of mc^{2}155. When 2 mM diamide is added to the mc^{2}155 liquid culture (Fig. 3B), the diamide is reduced steadily by mc^{2}155 until depletion at 8 h at which point exponential growth of mc^{2}155 commences. In contrast, when 2 mM diamide is added to the liquid culture with an initial cell density corresponding to 0.05 absorbance units, the diamide is reduced more slowly until depletion at 16 h at which point cell growth resumes but at a slow rate. In the presence of 6 mM diamide (Fig. 3C), the results are more striking. mc^{2}155 is able to reduce diamide by 12 h at which point exponential growth commences but T7 fails to grow. This failure to grow appears to correspond to the slow reduction of the diamide, with over half of the diamide still present in the oxidized form at 32 h in T7. As expected, these results indicate that T7 is unable to detoxify diamide at the same levels as mc^{2}155.

3.6. Sensitivity of T7 to other thiol oxidizing agents

Because iodoacetamide is also a thiol specific oxidizing agent that reacts with the –SH group in low molecular mass thiols, peptides and proteins to form thioethers, the sensitivity to this alkylating agent was determined by plating mc^{2}155 and T7 on solid media containing differing concentrations of iodoacetamide. In solid medium, T7 was able to grow on plates containing up to 0.01 mM iodoacetamide while mc^{2}155 was able to grow on plates containing up to 0.035 mM iodoacetamide indicating that T7 is at least 3.5-fold more sensitive to iodoacetamide. Moreover, addition of 0.01 mM iodoacetamide to liquid culture of mc^{2}155 and T7 caused a greater retardation of growth for T7 (Fig. 4B).

Table 2
Growth of mc^{2}155, T7 mutant, and T7 complements in solid media supplemented with diamide

Cell strain	Minimum concentration of inhibition (mM)
Mc^{2}155	10
T7	1
T7::pal0274	15
T7::pSodit0274	5

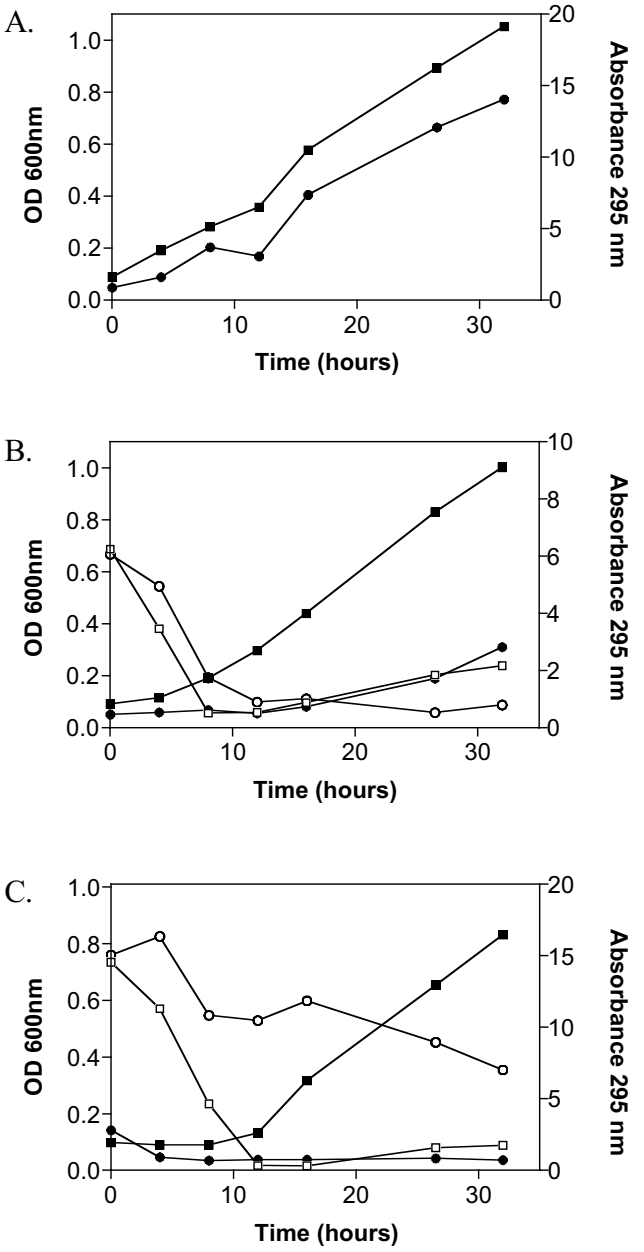

Fig. 3. *M. smegmatis* mc^{2}155 (closed squares) and T7 (closed circles) cell growth and the corresponding amounts of diamide remaining in the media (open squares for mc^{2}155 diamide levels and open circles for T7 diamide levels) (A) with no diamide present in the medium; (B) in medium containing 2 mM diamide; (C) in medium containing 6 mM diamide.

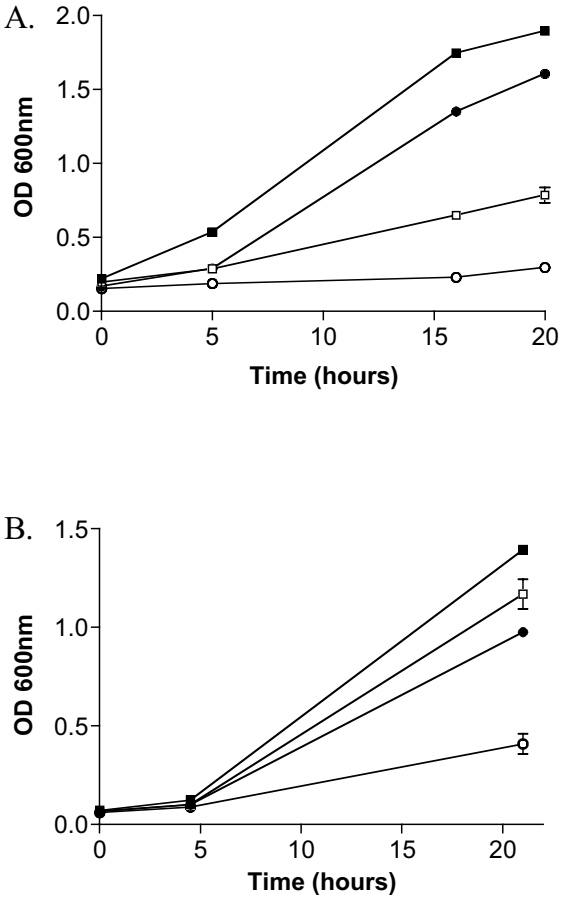

Fig. 4. Growth of *M. smegmatis* mc^{2}155 (closed squares) and T7 (closed circles) (A) with 0.01 mM CDNB present in the medium; (B) with 0.01 mM iodoacetamide present in the medium (open squares, mc^{2}155 with CDNB or iodoacetamide added; open circles, T7 with CDNB or iodoacetamide added).

Sensitivity to another thiol modifying agent, CDNB, which is customarily used in glutathione transferase assays as a substrate for glutathione transferase, was also determined for mc^{2}155 and T7. T7 was able to grow on plates containing up to 0.01 mM CDNB while mc^{2}155 was able to grow on plates containing up to 0.05 mM CDNB indicating that T7 is at least 5.0-fold more sensitive to CDNB. Addition of 0.01 mM CDNB to the liquid media of mc^{2}155 and T7 also caused a retardation of growth for both strains with T7 having a greater growth lag (Fig. 4A). Interestingly, *M. smegmatis* mc^{2}155 and T7 showed no difference in sensitivity to other redox cycling agents and the oxidizing agent cumene peroxide (data not shown).

3.7. M. tuberculosis *Rv0274 complements T7*

To complement the mutant T7, *M. tuberculosis* Rv0274 was cloned into *E. coli*/*Mycobacteria* shuttle vectors PALACE and pSODIT-2. Both vectors have genes for hygromycin resistance and replication origins suitable for maintenance in *E. coli* and mycobacteria. pSODIT-2 has a 193-bp promoter region from the *M. tuberculosis* superoxide dismutase (*sod*A) gene just upstream of the cloning site [17] while PALACE has an acetamidase promoter. Cloning into pSODIT-2 results in constitutive expression of the recombinant protein while in PALACE, the expression of the recombinant protein can be induced by the addition of acetamide to the media. When the complement T7::pSodit0274, containing the *M. tuberculosis* Rv0274 gene construct under the control of the constitutive promoter, was tested for diamide sensitivity, T7::pSodit0274 was partially able to reverse the mutant phenotype by growing on solid media with diamide concentrations of 7.5 mM or less. However, when the complement T7::pal0274, also containing the *M. tuberculosis* Rv0274 gene construct but under the inducible promoter, was tested for diamide sensitivity under inducing conditions, T7::pal0274 was able to grow in 15 mM diamide or less indicating that the overexpression of Rv0274 results in detoxification of diamide levels that are above and beyond that of the parent strain.

4. Discussion

In this report, we have identified a gene encoded by ORF Rv0274 of *M. tuberculosis* that is associated with protection against diamide induced stress in mycobacteria. A transposon mutant, T7, is unable to grow on levels of diamide on which the parent *M. smegmatis* can easily grow. The T7 mutant is complemented in its diamide sensitivity by the epichromosomal introduction of this gene. Unlike previously reported diamide sensitive mutants, this mutant is not sensitive to other oxidative stresses such as redox cycling radicals and organic peroxides.

The transposon insertion has been mapped upstream of the Rv0274 homolog in *M. smegmatis*, in a locus between Rv0273c and Rv0272c (Fig. 1C). This locus may contain the Rv0274 promoter and thus disruption in this region would result in a similar phenotype as a disruption in Rv0274 itself. Alternatively, this region may encode regulatory elements controlling Rv0274. Bioinformatic analysis (http://genolist.pasteur.fr/TubercuList/) revealed that Rv0272c encodes an unknown protein while Rv0273c is identified as a 'putative' transcriptional regulator. Thus, the mutation in Rv0273c might also be the reason for the increased sensitivity to diamide in this transposon mutant. Since this sensitivity was reversed when *M. tuberculosis* Rv0274 was expressed in the complemented strain, it is plausible that Rv0273c controls the transcription of Rv0274.

Diamide is a thiol oxidizing agent that perturbs the redox balance of the cell by oxidizing glutathione in glutathione containing organisms and, in the case of mycobacteria, mycothiol. Thus, the mode of action of diamide is depletion of low molecular mass thiol pools [21]. In glutathione containing organisms diamide reacts with gluta-

thione more rapidly than other thiol substrates having a $t_{1/2}$ of 1 s at mM concentrations [22]. When *E. coli* is exposed to diamide, there is reversible inhibition of cell growth at 0.3 and 0.6 mM diamide. The bacteriostatic effect of diamide may be due to the oxidization of glutathione to glutathione disulfide and the formation of mixed disulfides of glutathione with proteins [23]. The growth rate of *E. coli* recovers when the disulfides produced by diamide oxidation are reduced by glutathione reductase using NADPH generated from glucose metabolism. At higher concentrations, diamide causes lethal damage [24] presumably by depleting glutathione levels and thus inhibiting processes such as DNA synthesis and protein translation and initiation that depend on glutathione. Thus, the primary means of detoxification of diamide in *E. coli* appears to be glutathione. Indeed, mutants disrupted in the glutathione biosynthetic pathway *gsh*A, encoding γ-glutamylcysteine synthetase, *gsh*B, encoding glutathione synthetase, and *gor*, encoding glutathione reductase, are all diamide sensitive [8].

Mycobacteria do not produce glutathione but are still able to resist challenge by levels of diamide that are at least an order of magnitude higher than *E. coli* (Table 2 and Fig. 3) even though the mycothiol levels in mycobacteria are only two to four fold higher than those of glutathione in *E. coli*. In *E. coli*, glutathione levels have been estimated to be 4.5 μmol g^{-1} residual dry weight while mycothiol levels in *M. smegmatis* are fourfold higher at 10–19 μmol g^{-1} [4,6]. In solid media, wild-type *M. smegmatis* is able to grow on plates containing 10 mM diamide (Fig. 3) and although mycobacteria experience a dose dependent lag in growth on liquid media, *M. smegmatis* is easily able to grow on media containing 10 mM diamide (data not shown). Treatment with diamide most likely depletes mycothiol pools in mycobacteria in an analogous manner to the glutathione depletion in *E. coli* causing a growth lag. In T7, which has normal levels of mycothiol (data not shown), it appears that mycothiol is able to protect the cell against the effects of diamide by reacting with the diamide to a certain extent. However, complementation studies indicate that Rv0274 may play a role in detoxification of diamide. Complementation of the mutant with the *M. tuberculosis* Rv0274 cloned in an *E. coli*/*Mycobacterium* shuttle vector under control of a constitutive promoter results in a complement strain that shows partial recovery of the wild-type phenotype while complementation of the mutant with the *M. tuberculosis* Rv0274 cloned in a *E. coli*/*Mycobacterium* shuttle vector under control of an inducible promoter under inducing conditions results in a complemented strain that is being able to survive at levels of diamide that would be inhibitory for wild-type *M. smegmatis* indicating that Rv0274 is responsible for the diamide sensitivity of the mutant.

Bioinformatic analysis indicates that the protein encoded by Rv0274 belongs to a superfamily of metalloenzymes, VOC, of which the fosfomycin resistant glutathione transferase and a glutathione dependent glyoxalase are members. Another member of the VOC superfamily is the bleomycin resistance protein which binds and sequesters bleomycin and related compounds without degrading or transforming them. All the above proteins serve a similar protective function against toxins and antibiotics. Other members of the VOC superfamily include extradiol dioxygenases and methylmalonyl epimerases. Rv0274 is not likely to be a glyoxalase or an extradiol dioxygenase since the mutant does not show increased sensitivity to methylglyoxal and catechol, substrates for glyoxalases and extradiol oxygenases, respectively (data not shown). Rv0274 may instead encode a novel detoxification protein with a reaction mechanism similar to the enzymes of the VOC superfamily. Increased sensitivity of the mutant to iodoacetamide which forms thioethers with cysteines and CDNB, a glutathione transferase substrate that also reacts with cysteines, provides more evidence that this Rv0274 may encode a thiol specific detoxification protein. We are currently investigating this possibility.

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