Important role of the nucleotide excision repair pathway in *Mycobacterium smegmatis* in conferring protection against commonly encountered DNA-damaging agents

Krishna Kurthkoti, Pradeep Kumar, Ruchi Jain and Umesh Varshney

Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore 560012, India

Correspondence

Umesh Varshney
varshney@mcbl.iisc.ernet.in
or
uvarshney@gmail.com

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Mycobacteria are an important group of human pathogens. Although the DNA repair mechanisms in mycobacteria are not well understood, these are vital for the pathogen's persistence in the host macrophages. In this study, we generated a null mutation in the *uvrB* gene of *Mycobacterium smegmatis* to allow us to compare the significance of the nucleotide excision repair (NER) pathway with two important base excision repair pathways, initiated by uracil DNA glycosylase (Ung) and formamidopyrimidine DNA glycosylase (Fpg or MutM), in an isogenic strain background. The strain deficient in NER was the most sensitive to commonly encountered DNA-damaging agents such as UV, low pH, reactive oxygen species, hypoxia, and was also sensitive to acidified nitrite. Taken together with previous observations on NER-deficient *M. tuberculosis*, these results suggest that NER is an important DNA repair pathway in mycobacteria.

INTRODUCTION

As a part of the host's innate immune response launched to kill microbes, macrophages generate reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), and foster an environment that is low in pH. The oxyradicals and nitric oxide are highly soluble in lipids and diffuse through the microbial cell wall to cause lethal damage to DNA (Lancaster, 1997). Nitric oxide deaminates cytosine to uracil, adenine to hypoxanthine, and guanine to xanthine (Wink *et al.*, 1991). Oxidative stress results in formation of abasic sites, single- and double-stranded breaks in DNA and damage to nitrogenous bases such as the conversion of guanine to 7,8-dihydro-8-oxoguanine (8-oxoG). In addition, reaction of nitric oxide with molecular oxygen or superoxide anion forming peroxynitrite may induce inter-strand G–G cross-links and also polyamine DNA cross-links (Hartman *et al.*, 1986; Shapiro *et al.*, 1977). Failure to repair DNA damage is both cytotoxic and mutagenic. Thus DNA lesions are often subject to overlapping repair processes (Cooke *et al.*, 2003).

Mycobacterium tuberculosis, which resides in the host macrophages, is one of the most successful pathogens. It not only tolerates the hostile environment within macrophages but also survives environmental stresses like exposure to UV,

dehydration and low temperature during host exchanges (Manganelli *et al.*, 2004). Although the DNA damage response mechanisms in *M. tuberculosis* are not well understood, it is clear that for its persistence in the host, DNA repair strategies are vital for this pathogen (Mizrahi & Andersen, 1998).

The genome sequences of *M. tuberculosis* (Cole *et al.*, 1998), *Mycobacterium leprae* (Cole *et al.*, 2001; Smith *et al.*, 1997) and *Mycobacterium smegmatis* (<http://www.tigr.org>) have suggested that mismatch repair enzymes are missing in these organisms (Mizrahi & Andersen, 1998), and the role of RecA has been found to be minimal in mutation prevention (Boshoff *et al.*, 2003; Rand *et al.*, 2003). Thus, among the major DNA repair pathways, the base excision repair (BER) and the nucleotide excision repair (NER) pathways presumably contribute significantly to the maintenance of the genomic integrity in these bacteria. The high G+C content (~65 mol%) of the genomes of mycobacteria puts them at a greater risk of cytosine deamination (to uracil), and oxidative damage of guanosine (to 8-oxoG). The pathways that repair uracil and 8-oxoG are initiated by uracil DNA glycosylase (Ung or UDG; Lindahl, 1979; Duncan, 1981; Krokan *et al.*, 1997) and formamidopyrimidine DNA glycosylase (Fpg or MutM, Bailly *et al.*, 1989; Tchou *et al.*, 1991; Graves *et al.*, 1992), respectively, which are also two important BER enzymes in mycobacteria (Venkatesh *et al.*, 2003; Jain *et al.*, 2007). On the other hand, UvrB is a central player in NER (Skorvaga *et al.*, 2002).

Abbreviations: BER, base excision repair; NER, nucleotide excision repair; RNI, reactive nitrogen intermediates; ROS, reactive oxygen species.

Earlier studies have shown that a *uvrB*-deficient strain of *M. tuberculosis* (Darwin *et al.*, 2003) is markedly attenuated for survival in mice (Darwin & Nathan, 2005). However, there appear to have been no studies in which the relative importance of the DNA repair pathways in survival of mycobacteria has been compared in an isogenic strain background. Mutants of *M. tuberculosis* defective in *ung* or *fpg* have not been explored. Similarly, mutants of *M. smegmatis* defective in NER have been hitherto unavailable.

In this study, we generated a *uvrB*-deficient strain of *M. smegmatis*, and compared the relative fitness of this strain with those deficient in Ung (*ung*⁻) or Fpg (*fpg*⁻), the two important BER pathway enzymes, under commonly encountered conditions that damage DNA.

METHODS

Plasmids, media and growth conditions. Plasmids are listed in Table 1, and the details of various strains generated from *M. smegmatis* mc²155 (Snapper *et al.*, 1990) are listed in Table 2. *M. smegmatis* strains were grown in LB containing 0.2% (v/v) Tween 80 (LBT) or Middlebrook 7H9 (Difco) containing 0.2% (v/v) glycerol and 0.2% Tween 80. For growth on solid surfaces, 1.5% agar was included in the media. When specified, 7H10 medium (Difco) containing 0.5% (v/v) glycerol and 0.05% Tween 80 was used to obtain isolated colonies from glycerol stocks of various strains. Media were supplemented with hygromycin, gentamicin and kanamycin at 50, 5 and 50 µg ml⁻¹, respectively, as needed.

Disruption of the *uvrB* gene in *M. smegmatis*. *M. smegmatis uvrB* gene (Msm-*uvrB*, MSMEG_3816; <http://www.tigr.org>) was identified (Fig. 1a) by its homology to *M. tuberculosis uvrB* (Rv1633; Cole *et al.*, 1998) and the relevant part of it was PCR amplified using primers UvrB-Fp1 (5'-AGCCTGCAGTGGTGTGCTCGGCGC-3', which anneals ~160 bp downstream of the start codon in the UvrB ORF) and UvrB-Rp1 (5'-GCCAGCTCGAACTGCAGGTCG-3', which anneals ~70 bp upstream of the stop codon) and DyNAzyme EXT DNA polymerase (Finnzymes). PCR was carried out in a 50 µl reaction containing 1.5 U DyNAzyme EXT DNA polymerase

(Finnzymes), 250 ng *M. smegmatis* SN2 genomic DNA, 20 pmol of each primer, 3.5 mM MgSO₄, 5% DMSO, DyNAzyme EXT reaction buffer and 200 µM dNTPs. After initial denaturation for 4 min at 94 °C, 30 cycles of incubations were carried out at 94 °C for 1 min, 64 °C for 30 s and 72 °C for 2 min, followed by a final extension step at 72 °C for 10 min. The amplicon (~1.9 kb) was digested with *Pst*I and cloned into similarly digested pUC4k (Vieira & Messing, 1982), resulting in pUC4k-MsmUvrB, which was then digested with *Sph*I (internal to Msm-*uvrB*), blunt-ended with T4 DNA polymerase and ligated to a kanamycin cassette (~1.3 kb *Hinc*II fragment from pUC4k) to generate pUC4k-Msm-*uvrB::kan*^R. A 3.2 kb *Bam*HI fragment from this construct containing Msm-*uvrB::kan*^R was then mobilized into the *Bam*HI site of pPR27 (Pelicic *et al.*, 1997) to generate pPR-Msm-*uvrB::kan*^R, which was introduced into *M. smegmatis* mc²155 by electroporation (Hatfull & Jacobs, 2002) to generate the knockout strain using a protocol described before (Pelicic *et al.*, 1997; Venkatesh *et al.*, 2003).

Genomic blot analysis. Genomic DNAs (~2.5 µg) were digested with an excess of restriction enzymes (20 U), separated on a 0.7% agarose gel using TBE buffer, transferred (Reed & Mann, 1985) to nylon membrane (Biodyne B, Pall Gelman Laboratory) and subjected to hybridization (Vasanthakrishna *et al.*, 1997) with radiolabelled probe prepared by PCR using [α -³²P]dCTP and primers UvrB-Fp2 (5'-CGCACC GGCAAACCTTCG-3') and UvrB-Rp2 (5'-CGCGAA-TTCAGTCACGACGAC-3') (Sambrook *et al.*, 1989) using PCR product obtained by the same primers under standard conditions as template.

Generation of the *uvrB*-complemented strain. DNA oligomers UvrB-Fp3 (5'-CTATAAGCTTCGGGATCGGTATTG-3', which anneals ~244 bp upstream of the start codon in ORF) and UvrB-RP3 (5'-CGCTCGCTGTTTCGGTCACTTCAG-3', which anneals ~15 bp downstream of the termination codon) were used to PCR amplify Mtu-*uvrB* (Rv1633) from *M. tuberculosis* H37Rv genomic DNA (250 ng) using 1 U DyNAzyme EXT (Finnzyme) in a 50 µl reaction. The PCR conditions included an initial step of heating at 94 °C for 4 min followed by 30 cycles of incubations at 94 °C for 1 min, 47 °C for 30 s and 72 °C for 2 min 45 s and a final extension at 72 °C for 10 min. The amplicon (2.2 kb) was digested with *Hind*III, ethanol precipitated, phosphorylated with polynucleotide kinase, gel purified and cloned between the *Age*I (blunt-ended) and *Hind*III sites of pMV361 (*hyg*^R) (Stover *et al.*, 1991) to generate pMV-

Table 1. List of plasmids

Name	Sequence/relevant details	Reference
pUC4k (<i>kan</i> ^R)	Multicopy plasmid (<i>E. coli</i>) containing a <i>kan</i> ^R cassette	Vieira & Messing (1982)
pUC4k-Msm-UvrB	pUC4k harbouring a truncated ORF of <i>M. smegmatis uvrB</i> (MSMEG_3816)	This study
pUC4k-Msm- <i>uvrB::kan</i> ^R	pUC4k-Msm-UvrB where <i>uvrB</i> (MSMEG_3816) is disrupted with a <i>kan</i> ^R cassette	This study
pPR27 (Gm ^R)	<i>E. coli</i> -mycobacteria shuttle vector containing a temperature-sensitive <i>ori</i> (pAL5000) for replication in mycobacteria and a <i>sacB</i> marker for its counterselection	Pelicic <i>et al.</i> (1997)
pPR-Msm- <i>uvrB::kan</i> ^R	pPR27 containing <i>M. smegmatis uvrB::kan</i> in its <i>Bam</i> HI site	This study
pMV361 (<i>hyg</i> ^R)	Vector that replicates in <i>E. coli</i> and contains an <i>att</i> site for integration into the L5 <i>att</i> site in mycobacteria	Stover <i>et al.</i> (1991)
pMV-Mtu- <i>uvrB</i> (<i>hyg</i> ^R)	pMV361 harbouring <i>M. tuberculosis uvrB</i> (Rv1633)	This study
pPRmsUng	pPR27 harbouring <i>M. smegmatis ung</i>	Venkatesh <i>et al.</i> (2003)
pMV- <i>ung</i> (<i>hyg</i> ^R)	pMV361 harbouring <i>M. smegmatis ung</i>	This study
pTKmx	Shuttle vector harbouring ColE1 (<i>E. coli</i>) and pAL5000 (mycobacteria) origins of replication and <i>kan</i> ^R marker	Kenney & Churchward (1996)
pTK-Mtu- <i>fpg</i>	pTKmx harbouring <i>M. tuberculosis fpg</i>	Jain <i>et al.</i> (2007)

Table 2. *M. smegmatis* strains

Strain	Relevant details	Reference
mc ² 155	High-efficiency transformation strain	Snapper <i>et al.</i> (1990)
<i>uvrB</i> :: <i>kan</i> ^R	mc ² 155 where <i>uvrB</i> (MSMEG_3816) has been disrupted with <i>kan</i> cassette	This study
<i>fpg</i> :: <i>hyg</i> ^R	mc ² 155 where <i>fpg</i> has been replaced with <i>hyg</i> cassette	Jain <i>et al.</i> (2007)
<i>ung</i> :: <i>kan</i> ^R	mc ² 155 <i>ung</i> has been disrupted with <i>kan</i> cassette	Venkatesh <i>et al.</i> (2003)
WT	Designation used in this study for mc ² 155 harbouring pDK20 (<i>kan</i> ^R) at the L5 <i>att</i> site in the chromosome	Venkatesh <i>et al.</i> (2003)
<i>uvrB</i> ⁻	<i>M. smegmatis uvrB</i> :: <i>kan</i> ^R strain harbouring pMV361 (<i>hyg</i> ^R) at the L5 <i>att</i> site in the chromosome	This study
<i>ung</i> ⁻	<i>M. smegmatis ung</i> :: <i>kan</i> ^R strain harbouring pMV361 (<i>hyg</i> ^R) at the L5 <i>att</i> site in the chromosome	This study
<i>fpg</i> ⁻	<i>M. smegmatis fpg</i> :: <i>hyg</i> ^R strain harbouring pTKmx (<i>kan</i> ^R).	Jain <i>et al.</i> (2007)
<i>uvrB</i> ⁻ (L5att:: <i>uvrB</i>)	<i>M. smegmatis uvrB</i> :: <i>kan</i> ^R strain harbouring pMV-Mtu- <i>uvrB</i> (Rv1633) at the L5 <i>att</i> site in the chromosome	This study
<i>ung</i> ⁻ (L5att:: <i>ung</i>)	<i>M. smegmatis ung</i> :: <i>kan</i> ^R strain harbouring pMV- <i>ung</i> at the L5 <i>att</i> site in the chromosome	This study
<i>fpg</i> ⁻ (pTK-Mtu- <i>fpg</i>)	<i>M. smegmatis fpg</i> :: <i>hyg</i> ^R strain harbouring pTK-Mtu- <i>fpg</i>	Jain <i>et al.</i> (2007)

Mtu-*uvrB*, which was then introduced into *M. smegmatis* (*uvrB*::*kan*) by electroporation for its ectopic integration into the chromosome at the L5 *att* site to generate the *uvrB*⁻ (L5att::*uvrB*) strain. The vector alone (pMV361, *hyg*^R) was also electroporated into the *uvrB*::*kan* strain to generate the *uvrB*::*kan* L5att::pMV361 (referred to as *uvrB*⁻) strain. The strain designations have been defined in Table 2.

Generation of the *ung*-complemented strain. The *M. smegmatis ung* ORF along with ~700 bp of upstream and 663 bp of downstream sequences was released as ~2.1 kb fragment from pPRmsUng (Venkatesh *et al.*, 2003) by digestion with *Nco*I and *Xba*I, blunt-ended using Klenow fragment of DNA polymerase I and subcloned into *Pvu*II-digested pMV361 (Stover *et al.*, 1991) to generate pMVMsm-*ung* (*hyg*^R), which was then introduced into *M. smegmatis ung*::*kan* by electroporation to generate *M. smegmatis ung*::*kan* (L5att::*ung*), referred to as [*ung*⁻ (L5att::*ung*)], containing an ectopic copy of *ung* at the L5att site (Table 2).

Assay for UV sensitivity. An early-exponential-phase culture (OD₅₉₅ ~0.4) of *M. smegmatis* was serially diluted 1:5 in LBT in a 96-well plate. Samples from the third dilution onwards were spotted on LBT agar plates with a 48-pronged spotter (Sigma) and plates were exposed to UV C in a biosafety cabinet to UV intensities of 0, 30 or 60 J m⁻² (as measured with a dosimeter), then incubated at 37 °C for 3 days.

Determination of mutation rates. Mutation rates were determined according to David (1970). Briefly, isolated colonies of the various *M. smegmatis* strains were grown to saturation in 7H9 medium containing 0.2 % glycerol, 0.1 % Tween 80 and 10 % ADC (Difco) along with the appropriate antibiotics for 48 h. Subsequently, for each strain, 12 independent tubes containing 2 ml each of the same medium but without antibiotics were inoculated with ~1500–2000 bacteria by diluting from the freshly prepared saturated cultures, and grown for 6 days at 37 °C with vigorous shaking. Absence of spontaneous mutants in the inoculum (used to develop the 6 day cultures) was confirmed by plating an equivalent aliquot of the culture on 7H10 containing 0.5 % glycerol, 10 % OADC (Difco) and 100 µg rifampicin ml⁻¹. At the end of the 6 day growth, small aliquots from cultures were used to determine total viable counts by dilution plating on LBT agar, and the cells from the remainder of the cultures were harvested and plated on 7H10 containing 0.5 % glycerol, 10 % OADC and 100 µg

rifampicin ml⁻¹. The plates were incubated for 3–5 days and the colonies appearing on the plates were counted. The mutation rates for each replicate of the strain were determined and mean mutation rates were calculated (David, 1970).

Effect of acidified sodium nitrite. Isolated colonies of *M. smegmatis* strains were grown in triplicate in 7H9 medium with appropriate antibiotics to saturation (55–60 h). The cultures were serially diluted 1:100 in LBT (pH 5.5) with 0.5 % (w/v) BSA, supplemented with 0, 0.5, 1.5 or 2.5 mM freshly prepared sodium nitrite (Merck, India), seeded in the microtitre wells of honeycomb plates and incubated in a Bioscreen C kinetic growth reader at 37 °C with constant shaking at maximum amplitude. Growth was monitored by the machine, which measured OD₆₀₀ at 3 h intervals for 39 h.

Effect of hydrogen peroxide on bacterial growth. The effect of H₂O₂ on growth was determined in a manner similar to that of acidified sodium nitrite except that the saturated cultures of *M. smegmatis* strains were diluted in LBT with 0.5 % BSA containing 0, 0.5, 1.0 or 1.5 mM H₂O₂ (Calbiochem).

Effect of hypoxic stress on bacterial survival. *M. smegmatis* strains were grown to an OD₆₀₀ of 0.5–0.6 (~1.5–2.0 × 10⁸ c.f.u. ml⁻¹) in Dubos medium with 10 % ADC and diluted 1:100 in triplicate in screw-cap flat-bottom culture tubes containing 20 ml of the same medium and 10 ml of air space (headspace ratio 0.5; Dick *et al.*, 1998). The tubes were sealed and the cultures were grown with slow stirring using a multipoint magnetic board at 37 °C for 10 days. Total viable counts of these cultures were determined by dilution plating on LBT agar. The cultures (from hypoxic conditions) were also diluted 1:100 in triplicate in fresh Dubos medium (with ADC) and recovered aerobically under vigorous shaking conditions at 37 °C. Total viable counts of the recovered cultures were determined at 3, 6, 12 and 24 h by dilution plating and plotted as log₁₀ c.f.u. ml⁻¹.

RESULTS

uvrB gene disruption in *M. smegmatis*

The nucleotide sequence of the *M. smegmatis uvrB* gene (MSMEG_3816) was retrieved from the *M. smegmatis*

sequence available at the TIGR website by BLAST search using the *M. tuberculosis* *uvrB* (Rv1633) sequence as query. The two were found to be highly conserved at both the amino acid level (~95 % similarity and ~ 92 % identity) and the nucleotide sequence level (85.3 %). The genomic contexts of *uvrB* in *M. tuberculosis* and *M. smegmatis* are shown in Fig. 1(a). The *M. smegmatis* *uvrB* knockout strain was generated by insertion of a kanamycin resistance (*kan^R*) cassette (~1.3 kb) within the ORF of *uvrB* (Fig. 1b). The disruption of chromosomal *uvrB* was confirmed by genomic blot analysis. As expected (Fig. 1b), a *ScaI*/*Bam*HI double digest resulted in detection of a ~2.5 kb fragment (Fig. 1c, lane 1) from the wild-type and a ~3.8 kb fragment from the knockout strains (lanes 2 and 3). The *NarI* digests also showed the expected fragment sizes of ~2.3 kb for the wild-type (lane 4) and ~3.6 kb for the knockout strain (lanes 5 and 6). These results confirmed the authenticity of the *uvrB* gene knockout. Assays of Fpg and Ung activity (performed as previously described: Venkatesh *et al.*, 2003; Jain *et al.*, 2007) confirmed that neither of these activities was disrupted in the *uvrB* deletion mutant (data not shown).

Analysis of the mutation rates

To investigate the effect of the UvrB deficiency (deficiency of NER) on the mutation rates of the strain and to compare it with those of strains deficient in the BER pathways (*ung⁻* and *fpg⁻*) in *M. smegmatis*, we determined the mutation rates of the bacterial cultures by scoring for appearance of rifampicin-resistant colonies in the various strains. As shown in Table 3, the loss of Fpg or UvrB proteins resulted in a ~2.5-fold increase in the mutation rates compared to the wild-type strain. On the other hand, under the same conditions, the deficiency of Ung resulted in a ~9-fold increase in the mutation rate.

Analysis of UV sensitivity

Earlier studies showed that a *M. tuberculosis* strain deficient in *uvrB* did not grow following exposure to UV (Darwin & Nathan, 2005). We compared the sensitivity to UV radiation of *M. smegmatis* strains deficient in DNA repair enzymes. All the strains showed similar growth when not exposed to UV (Fig. 2, 0 J m⁻²). However, the strain deficient in *uvrB* was highly sensitive to UV C radiation

Fig. 1. Generation and characterization of the *M. smegmatis* *uvrB::kan* strain. (a) Schematic representation (not to scale) of the genomic context of *uvrB* in *M. smegmatis* (*Msm*) between positions 3877922 and 3889576, and comparison with that from the *M. tuberculosis* (*Mtu*) genome. Details of various annotated genes in *Mtu* and *Msm* genomes are as follows: Rv1629, *polA*; Rv1630, *rspA*; Rv1631, *coaE*; Rv1632c, hypothetical protein; Rv1633, *uvrB*; Rv1634, possible drug efflux membrane protein; Rv1635c, probable conserved transmembrane protein; Rv1636, iron-regulated conserved hypothetical protein TB15.3; Rv1637c, hypothetical protein; Rv1638, *uvrA*; MSMEG_3808, *uvrA*; MSMEG_3810, probable hydrolase (HMM PF00753 family); MSMEG_3811, probable universal protein stress family protein; MSMEG_3812, acyl-CoA thioesterase; MSMEG_3813, NAD-dependent epimerase/dehydratase; MSMEG_3814, tRNA-Cys; MSMEG_3815, possible drug efflux membrane protein; MSMEG_3816, *uvrB*; MSMEG_3817, conserved hypothetical protein; MSMEG_3818, thermolabile glutaminase; MSMEG_3819, conserved hypothetical protein. (b) Schematic drawing of the wild-type and disrupted (*uvrB::kan*) *uvrB* loci in *M. smegmatis*, showing various restriction endonuclease sites. The location of the *uvrB*-specific probe prepared in the presence of [α -³²P]dCTP by a PCR-based method marked is indicated by the dotted line labelled 'Probe' above the wild-type locus. (c) Genomic blot analysis of *M. smegmatis* strains. Sizes of DNA bands (sizes in kb) are as indicated.

Table 3. Effect of deficiency in DNA repair enzymes on mutator phenotypes

Strain	$10^{-9} \times$ Total no. of viable bacteria plated*	$10^{10} \times$ Mutation rate†	Fold increase
WT	8.9 ± 0.91	1.83	1.0
<i>ung::kan</i>	8.2 ± 0.54	16.9	9.2
<i>fpg::hyg</i>	7.9 ± 1.8	4.64	2.5
<i>uvrB::kan</i>	8.3 ± 0.517	4.73	2.6

*Mean $\pm$ SD of the total viable counts (determined by dilution plating of an aliquot) of bacteria in the 6 day cultures used to plate on rifampicin plates.

†Mutation rates were calculated according to David (1970) using the equation $a = 2 \ln 2 [(M_t/N_t) - (M_0/N_0)]/n$, where a is the mutation rate, n the number of generations, M_t the number of Rif^R colonies obtained from the 6 day culture (Methods), N_t the number of bacteria in the 6 day culture, M_0 the number of Rif^R colonies in the starter culture used to make the 6 day culture, and N_0 the number of bacteria in the starter culture used to make the 6 day culture. The value of n was calculated as $(\log N_t - \log N_0)/0.301$. As the value of M_0 was zero, the simplified equation $a = 2 \ln 2 (M_t/N_t)/n$ was used to calculate mutation rates. Mutation rates were calculated for all 12 replicates of the 6 day cultures, and the mean mutation rates shown were calculated from 10 replicates, eliminating the two with the highest and the lowest rates.

even at 30 J m^{-2} . The other strains showed no visible growth defect even at the higher dose of 60 J m^{-2} . Based on earlier studies in other organisms (Czeczot *et al.*, 1991; Scott *et al.*, 1999; Huang *et al.*, 2006), it was not unexpected that the deficiency of Ung and Fpg did not result in any increased sensitivity of *M. smegmatis* to UV C radiation. However, these observations highlight that the NER pathway provides the major means to protect *M. smegmatis* against DNA damage caused by UV radiation. As a control, integration of a single copy of the *uvrB* gene (from *M. tuberculosis*) at the L5 *att* site in the knockout strain rescued the phenotype (bottom row in Fig. 2), suggesting

that the UV sensitivity of the strain was due to UvrB deficiency and not an indirect consequence of chromosomal alteration at the *uvrB* locus.

Effect of acidified sodium nitrite on the growth of *M. smegmatis* strains

Under acidic conditions, sodium nitrite generates nitrous acid, which decomposes to generate a range of RNI (Stuehr *et al.*, 1989; O'Brien *et al.*, 1994). RNI can cause deamination of various bases in DNA or could react with oxygen to form peroxynitrite, causing oxidative damage to cells. Earlier work (Venkatesh *et al.*, 2003; Darwin & Nathan, 2005) has shown that mycobacterial strains deficient in Ung or UvrB (*M. smegmatis* and *M. tuberculosis*, respectively) are susceptible to nitric oxide stress. We compared the effect of nitric oxide on strains of *M. smegmatis* deficient in Fpg, Ung, and UvrB at initial culture pH of 5.5. Representative growth curves are presented in Fig. 3. As seen in Fig. 3(a), the medium pH of 5.5 itself had an effect on the growth profile of the strains, and the growth of the strain deficient in NER was the most compromised. While the supplementation of the medium with increasing amounts of sodium nitrite (Fig. 3b–d) resulted in additional compromise in the growth of the NER-deficient strain, the effect of the reagent on the *ung*[−] strain was more pronounced (e.g. compare its growth with that of the wild-type strain in panels a and c). The effect of the reagent on the *fpg*[−] strain was the least pronounced. Thus, the *ung*[−] and *uvrB*[−] but not the *fpg*[−] strains, under the conditions used, are sensitive to growth in acidified nitrite. As a control, at the highest concentration of the reagent used, the *ung*[−], *uvrB*[−] and *fpg*[−] strains grew like the wild-type parent when complemented with, respectively, an ectopically inserted copy of *ung* or *uvrB* at the L5 *att* site in the chromosome, and *fpg* on a vector, pTK-Mtu-*fpg* (Fig. 3e). It may be noted that the pH of the medium changes to neutral during the mid-exponential phase of the culture.

Fig. 2. UV sensitivity assay using *M. smegmatis* strains [WT, *ung*[−], *fpg*[−], *uvrB*[−] and *uvrB*[−](L5att::*uvrB*)]. Various dilutions (as indicated) of early-exponential-phase cultures were spotted onto LBT agar and either not exposed (0 J m^{-2}) or exposed to 30 J m^{-2} or 60 J m^{-2} of UV C radiation. The plates were then incubated at 37°C for 3 days and imaged.

Fig. 3. Effect of acidified NaNO₂ on growth. Saturated cultures of various strains of *M. smegmatis* [WT, *ung*⁻, *ung*⁻(L5att::*ung*), *uvrB*⁻, *uvrB*⁻(L5att::*uvrB*), *fpg*⁻ and *fpg*⁻(pTK-Mtu-*fpg*)] were diluted 100-fold in LBT with 0.5% (w/v) BSA, pH 5.5 containing no NaNO₂ (a), 0.5 mM NaNO₂ (b), 1.5 mM NaNO₂ (c) or 2.5 mM NaNO₂ (d, e) and grown at 37 °C for 39 h in a Bioscreen C kinetic growth reader in microtitre plates. Growth curves were prepared from three independent colonies for each strain; means ± SD are plotted. Analysis of the total viable counts during the exponential and stationary phases of the growth (data not shown) revealed that the OD₆₀₀ measurements of the various strains gave a true representation of their viability.

Effect of hydrogen peroxide on the growth of *M. smegmatis* strains

We previously observed that *M. smegmatis* deficient in *fpg* is susceptible to H₂O₂ (Jain *et al.*, 2007). Such analyses have not been carried out for the strains deficient in Ung and UvrB. As shown by the representative data in Fig. 4, all the strains showed similar growth kinetics in the absence of H₂O₂ (Fig. 4a). Addition of the increasing amounts of H₂O₂ (0.5, 1 and 1.5 mM) to the growth medium revealed that the *NER*-deficient strain was the most sensitive to the reagent, and at 1.5 mM H₂O₂ it failed to grow (Fig. 4b–d). At 1 mM H₂O₂, a small effect could also be detected for the *fpg*⁻ strain but not the *ung*⁻ strain (Fig. 4c). However, at 1.5 mM H₂O₂, growth of both the *fpg*⁻ and the *ung*⁻ strains was also severely impaired. As a control, growth of the complemented strains was similar to that of the wild-type strain even at the highest concentration of the reagent used (Fig. 4e). These observations show that among the strains tested, the *uvrB*⁻ strain is highly susceptible to H₂O₂.

Effect of hypoxic stress on growth and survival of the strains

During latency, pathogenic mycobacteria survive under hypoxia. Wayne has defined *in vitro* growth conditions to mimic hypoxia using sealed culture tubes with a defined

empty space above the medium (Wayne & Lin, 1982; Wayne & Hayes, 1996). Earlier reports (Dick *et al.*, 1998; Mayuri *et al.*, 2002) have described adaptation of these growth conditions for *M. smegmatis* and shown that several physiological changes that the bacterium undergoes during such a growth are common to those encountered by *M. tuberculosis* under the same conditions. Considering that hypoxic conditions are known to induce DNA damage (Moller *et al.*, 2001; Grishko *et al.*, 2001) it was of interest to subject the various DNA-repair-deficient *M. smegmatis* strains to the hypoxic growth model of Wayne. As shown in Fig. 5(a), evaluation of the total viable counts of the various cultures at the end of the growth regimen revealed that in comparison with the WT strain the *uvrB*⁻ strain was the least fit for survival under hypoxia. Although the *ung*⁻ strain also showed impaired survival under these conditions, the *fpg*⁻ strain was unaffected. Importantly, complementation of the *uvrB*⁻ and the *ung*⁻ strains with the *uvrB* and *ung* genes restored their survival level to the WT level. The complementation of the *fpg*⁻ strain with a plasmid-borne copy of *fpg* resulted in a slight decrease in total viable counts (in comparison to the *fpg*⁻ strain). The reasons for this are not clear. Notwithstanding this observation, it is clear that of all the strains, the *uvrB*⁻ strain is most sensitive to hypoxia as well.

To further analyse the effect of hypoxia on the DNA-repair-deficient strains, we subcultured the strains after

Fig. 4. Effect of H_2O_2 on growth. Saturated cultures of various strains of *M. smegmatis* [WT, *ung*⁻, *ung*⁻(L5att::*ung*), *uvrB*⁻, *uvrB*⁻(L5att::*uvrB*), *fpg*⁻ and *fpg*⁻(pTK-Mtu-*fpg*)] were diluted 100-fold in LBT with 0.5% (w/v) BSA. H_2O_2 was either not added (a), or added at 0.5 mM (b), 1.0 mM (c) or 1.5 mM (d, e). Cultures were grown at 37 °C for 39 h in a Bioscreen C kinetic growth reader in microtitre plates. Growth curves were done from three independent colonies for each strain; means $\pm$ SD are plotted. As was the case for the experiments in Fig. 3, the OD₆₀₀ measurements represented true viable counts.

their release from hypoxia and monitored them for their adaptability to growth under normal conditions. Interestingly, while the *ung*⁻ and the *fpg*⁻ strains recovered

well, the *uvrB*⁻ strain was highly compromised even during its recovery phase (Fig. 5b). As expected, all the complemented strains recovered as well as the WT strain.

Fig. 5. Effect of hypoxia on survival and recovery of *M. smegmatis* strains using Wayne's model of *in vitro* growth. Mid-exponential-phase cultures of various *M. smegmatis* strains (in triplicate) were grown to OD₆₀₀ 0.5–0.6 in Dubos medium with ADC and diluted 1:100 in the same medium for growth in sealed screw-cap flat-bottom culture tubes at 37 °C for 10 days. Viable counts of these cultures were determined by dilution plating on LBT agar. Mean values ($\pm$ SD) for each strain are shown in (a). The cultures (after release from hypoxia) were also diluted 1:100 in triplicate in fresh Dubos medium with ADC and recovered aerobically under vigorously shaken conditions at 37 °C. Viable counts of the recovered cultures were determined at 3, 6, 12 and 24 h by dilution plating and mean values ($\pm$ SD) are plotted in (b).

DISCUSSION

M. tuberculosis exists in the host in either a phase of active multiplication or a phase where it remains latent. Physiological differences between the two phases suggest differential requirements/utilization of the essential biochemical processes during these phases. During the state of latency, while a large number of cellular processes could be downregulated, a compromise in DNA repair processes, essential to maintain the integrity of the genetic blueprint, could prove detrimental to survival. Considering that in the host, *M. tuberculosis* is exposed to RNI and ROS which are known to damage DNA, a deficiency in the DNA repair capacity of the bacterium would result in accumulation of mutations in its genome and failure of its reactivation. A number of available drugs target mycobacteria in the active multiplication phase. As development of drugs to target the bacilli in the latent phase is a priority, it is crucial to assess the relative importance of different repair pathways in mycobacteria to enable their exploitation as a possible target. To perform such studies, it is desirable that mutations in different repair pathways are available in an isogenic strain background.

As expected for the G+C-rich genomes of *M. tuberculosis* and *M. smegmatis*, the Ung- and Fpg-mediated repair pathways represent two of the crucial BER pathways in these bacteria (Venkatesh *et al.*, 2003; Jain *et al.*, 2007). Neither of these repair pathways has been investigated in *M. tuberculosis*. On the other hand, while the consequences of NER deficiency have been investigated previously (Darwin & Nathan, 2005) in a transposon insertion mutant of *M. tuberculosis* (Darwin *et al.*, 2003), its significance had not been reported in *M. smegmatis*. In this study, we have generated an *uvrB* knockout strain of *M. smegmatis* to allow a direct comparison of the significance of different DNA repair pathways in mycobacteria.

We previously reported an increase in mutation frequencies of about 8- and 3.8-fold in Ung- and Fpg-deficient strains, respectively (Venkatesh *et al.*, 2003; Jain *et al.*, 2007). As mutation frequency analysis can be influenced by various factors, for a comparative analysis of the mutator phenotypes, in this study we carried out mutation rate analyses (David, 1970). This analysis revealed that Ung, Fpg and UvrB deficiencies resulted in a ~9-, 2.6- and 2.5-fold increase, respectively, in the mutation rates above the WT reference (Table 3). However, compared with Ung- and Fpg-deficient strains, the UvrB-deficient strain was more sensitive to exposure to UV, low pH, H₂O₂ and hypoxia (Figs 2, 3, 4 and 5), emphasizing a vital role of NER in mycobacteria. It may be noted that the susceptibility of *M. smegmatis* to H₂O₂ is in contrast to the observations made with a *uvrB*-deficient strain of *M. tuberculosis* (Darwin & Nathan, 2005). The reasons for this observed difference are unclear, but it may well be that the culture age and the growth conditions play a role in susceptibility to H₂O₂ (e.g. the presence of catalase, a

component of OADC supplement, in the culture medium of *M. tuberculosis* may neutralize H₂O₂). Also, while mutational analysis with the NER-deficient strain of *M. tuberculosis* has not been carried out, our observations on the remaining phenotypes of NER-deficient *M. smegmatis* reinforce the observations made with the *uvrB* transposon mutant of *M. tuberculosis* isolated by Darwin *et al.* (2003) and then extensively characterized by Darwin & Nathan (2005). Further, while a direct comparison of the effects of the BER and NER such as that carried out in this study is not available, studies in *Escherichia coli* have shown that NER deficiency resulted in only a marginal increase in mutation frequencies (Pienkowska *et al.*, 1993). Interestingly, Hall (1995) showed that NER deficiency in a tryptophan auxotroph of *E. coli* resulted in a >100-fold increase in mutation rates of *trpA46* to *trpA*⁺ under tryptophan starvation. However, under tryptophan-sufficiency conditions the mutation rates were not affected by NER deficiency.

It has also been shown that NER plays a major role in repair of 8-oxoG, cyclo-dA and cyclo-dG (Scott *et al.*, 1999; Kuraoka *et al.*, 2000). In fact, overlapping functions of Fpg and NER have also been suggested by other studies (Kow *et al.*, 1990; Asad *et al.*, 2000). Deamination of guanine to xanthine can also result in depurination to form abasic sites in DNA (Tamir *et al.*, 1996), which could be substrates for NER (Lin & Sancar, 1989). These observations support our findings on the sensitivity of NER-deficient bacteria to H₂O₂.

It is significant that *uvrA* and *uvrB* are induced in mycobacteria upon treatment with DNA-damaging agents like mitomycin C as well as upon infecting human macrophages (Rand *et al.*, 2003; Graham & Clark-Curtiss, 1999). Thus, NER seems to be central to DNA repair mechanisms in mycobacteria. Taken together, these and earlier observations (Darwin & Nathan, 2005) show that mycobacteria deficient in NER could be a good candidate for development of attenuated strains, and development of inhibitors against NER proteins may allow an efficient method of killing mycobacteria.

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