Synergistic effects of UdgB and Ung in mutation prevention and protection against commonly encountered DNA damaging agents in *Mycobacterium smegmatis*

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The incorporation of dUMP during replication or the deamination of cytosine in DNA results in the occurrence of uracils in genomes. To maintain genomic integrity, uracil DNA glycosylases (UDGs) excise uracil from DNA and initiate the base-excision repair pathway. Here, we cloned, purified and biochemically characterized a family 5 UDG, UdgB, from *Mycobacterium smegmatis* to allow us to use it as a model organism to investigate the physiological significance of the novel enzyme. Studies with knockout strains showed that compared with the wild-type parent, the mutation rate of the *udgB*[−] strain was approximately twofold higher, whereas the mutation rate of a strain deficient in the family 1 UDG (*ung*[−]) was found to be ~8.4-fold higher. Interestingly, the mutation rate of the double-knockout (*ung*[−]*udgB*[−]) strain was remarkably high, at ~19.6-fold. While CG to TA mutations predominated in the *ung*[−] and *ung*[−]*udgB*[−] strains, AT to GC mutations were enhanced in the *udgB*[−] strain. The *ung*[−]*udgB*[−] strain was notably more sensitive to acidified nitrite and hydrogen peroxide stresses compared with the single knockouts (*ung*[−] or *udgB*[−]). These observations reveal a synergistic effect of UdgB and Ung in DNA repair, and could have implications for the generation of attenuated strains of *Mycobacterium tuberculosis*.

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INTRODUCTION

Pathogenic mycobacteria encounter a number of physiological stresses within host macrophages. To kill the pathogen, the host macrophages generate reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI). RNI, which include nitric oxide and its derivatives, can deaminate cytosine to uracil, adenine to hypoxanthine (Hx), and guanine to xanthine in DNA as well as in the free nucleotide pool (Wink *et al.*, 1991). As maintenance of genomic integrity is essential for the survival of an organism, pathogens have evolved multiple repair pathways to mend DNA damage.

Deamination of C to U occurs at a high frequency in DNA even under normal physiological conditions, and can be increased by environmental mutagens (Lindahl, 1993). Failure to repair this damage prior to replication leads to accumulation of CG to TA mutations in subsequent

generations. The A to Hx reaction is about 10 times slower than that of C to U. Hx pairing with C during replication leads to AT to GC mutation. A number of base excision repair (BER) pathways, dedicated to preventing such mutations, play a crucial role in restoration of genomic integrity. The genome sequences of *Mycobacterium tuberculosis* and *Mycobacterium smegmatis* have revealed that they lack the mismatch repair and the very short patch repair pathway enzymes (Cole *et al.*, 1998; Mizrahi & Andersen, 1998). Furthermore, RecA-mediated repair also does not appear to play a significant role in mutation prevention in mycobacteria (Boshoff *et al.*, 2003; Rand *et al.*, 2003). Thus, the base- and nucleotide-excision repair pathways must play significant roles in maintaining genomic integrity in these bacteria (Kurthkoti *et al.*, 2008).

Uracil DNA glycosylases (UDGs) are a class of enzymes that catalyse the first step in uracil excision repair in DNA. Based on their substrate specificities and the two highly conserved sequence motifs (motif A and motif B) crucial in catalysis, UDGs have been classified into five families (Sartori *et al.*, 2002). The two UDGs that are the focus of this study belong to family 1 and family 5. The family 1 UDGs, also known as Ung, excise U from both ssDNA and dsDNA, and possess two highly conserved sequences,

Abbreviations: Hx, hypoxanthine; RNI, reactive nitrogen intermediates; ROS, reactive oxygen species; RRDR, rifampicin resistance determining region; UDG, uracil DNA glycosylase.

A supplementary table, listing oligodeoxyribonucleotides and plasmids used, is available with the online version of this paper.

GQDPY (motif A) and HPSPLS (motif B) (Krokan *et al.*, 1997). Family 5 UDGs, also known as UdgB, are restricted to a limited number of organisms that survive in extreme habitats (Sartori *et al.*, 2002; Starkuviene & Fritz, 2002; Wanner *et al.*, 2009), excise uracil from dsDNA and possess conserved sequences, GLAPA and HPSPLNV (HPSQQN in *M. tuberculosis*) as motif A and motif B, respectively. In addition to uracil, the UdgB proteins excise 5-hydroxymethyl-uracil, ethenocytosine, 5-fluorouracil and Hx from dsDNA (Srinath *et al.*, 2007; Sartori *et al.*, 2002). The *M. tuberculosis* UdgB has also been shown to rescue the *ung*[−] phenotype of *Escherichia coli* (Srinath *et al.*, 2007). However, the unavailability of either *ung*[−] or *udgB*[−] strains of *M. tuberculosis* has severely hampered analysis of the physiological significance of UDGs in this important pathogen.

Here, we identified the MSMEG_5031 from *M. smegmatis* as the homologue of *MtuUdgB*, overexpressed and purified the homologue from an *ung*[−] strain of *E. coli*, and carried out its biochemical characterization to validate *M. smegmatis* as a tractable model to study the physiological significance of UdgB in mycobacteria. We then generated *udgB* knockouts in *M. smegmatis* (mc²155) and its *ung*[−] derivative to study the effects of the two UDGs on mutation rates, mutation spectra and growth under conditions that mimic physiological stresses.

METHODS

DNA oligomers and their 5'-end labelling. DNA oligomers (Supplementary Table S1) were obtained from Microsynth, purified using Sep-Pak (C18) cartridges (Waters), quantified by measurement of A₂₆₀ and diluted in water at 0.1 n A₂₆₀ units per ml (where *n* is the number of nucleotides in the oligomer) to obtain a strand concentration of 10 nmol ml^{−1}. DNA oligomers (10 pmol) were 5' ³²P end-labelled using 10 µCi (370 kBq) [γ-³²P]ATP (6000 Ci mmol^{−1}; 222 000 GBq mmol^{−1}) and T4 polynucleotide kinase, and purified on Sephadex G50 minicolumns (Varshney & van de Sande, 1991). GU9, a tetra-loop hairpin oligomer containing uracil in the stem region, was used as dsDNA substrate.

Plasmids, media and growth conditions. Plasmids are listed in Supplementary Table S1, and the details of the various strains

generated from *M. smegmatis* mc²155 (Snapper *et al.*, 1990) are provided in Table 1. *M. smegmatis* strains were grown in Luria-Bertani (LB) medium 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 (Hyg), gentamicin (Gen) and kanamycin (Kan) at 50, 5 and 50 µg ml^{−1}, respectively, as needed.

Cloning of *MsmUdgB* in pET14b and pTrc99C. The *M. smegmatis* *udgB* gene (MSMEG_5031; <http://www.jcvi.org/>) was identified by its homology to *M. tuberculosis* *udgB* (Rv1259; Cole *et al.*, 1998). The ORF of *MsmUdgB* (MSMEG_5031, nucleotide positions 5 127 697–5 126 627) was amplified by PCR from *M. smegmatis* mc²155 genomic DNA using a forward *MsmUdgB*-Fp and a reverse *MsmUdgB*-Rp primer containing *NdeI* and *HindIII* sites, respectively, and DyNAzyme EXT (Finnzymes). The reaction was heated at 94 °C for 4 min, followed by 30 cycles of incubation at 94 °C for 1 min, 64 °C for 30 s and 72 °C for 1 min 10 s. The PCR product (~1 kb) was digested with *NdeI* and *HindIII*, cloned into similarly digested pET14b (Novagen) to generate pET*MsmUdgB*, and confirmed by DNA sequence analysis. This construct appended a 20 aa (MGSSHHHHHHSSGLVPRGSH) pre-sequence to the N-terminal region of *MsmUdgB*. Subsequently, the *MsmUdgB* DNA sequence (along with the pre-sequence) was excised from pET*MsmUdgB* with *NcoI* and *HindIII* and mobilized into similarly digested pTrc99C (GE Healthcare) to generate pTrc*MsmUdgB*.

Overexpression and purification of *MsmUdgB*, and UDG assays. Colonies of *E. coli* BW310 (*ung*[−]) harbouring pTrc*MsmUdgB* were grown under shaking conditions in LB medium (8 l) containing ampicillin (100 µg ml^{−1}) and ferric chloride (0.01 %, w/v) to OD₆₀₀ ~0.6 at 37 °C, supplemented with 0.5 mM IPTG, and shaken further for 4 h at 37 °C. Cells were harvested by centrifugation at 4 °C, suspended in buffer A [20 mM HEPES (pH 7.5), 10 % glycerol (v/v), 100 mM NaCl and 2 mM β-mercaptoethanol], ultrasonicated and subjected to centrifugation at 14 000 g for 20 min at 4 °C. The supernatant was loaded onto an Ni-NTA column (5 ml, Pharmacia Biotech) equilibrated with buffer A. The column was washed with 20 ml buffer A containing 10 mM imidazole, followed by another wash with buffer A containing 10 mM imidazole and 500 mM NaCl. *MsmUdgB* was then eluted with a gradient of 10–1000 mM imidazole in buffer A containing 500 mM NaCl. The fractions containing *MsmUdgB* were identified by SDS-PAGE (15 %), pooled and dialysed against buffer A. Subsequently, the UdgB preparation was passed through a Mono-S

Table 1. *M. smegmatis* strains

<i>M. smegmatis</i> strain	Relevant details	Reference or source
Wild-type	High-efficiency transformation strain <i>M. smegmatis</i> mc ² 155	Snapper <i>et al.</i> (1990)
Wild-type (Kan)	Designation used in this study for <i>M. smegmatis</i> mc ² 155 harbouring pDK20 (Kan ^R) at the L5 <i>att</i> site in the chromosome	Venkatesh <i>et al.</i> (2003)
<i>udgB</i> [−]	<i>M. smegmatis</i> mc ² 155 in which the <i>udgB</i> (MSMEG_5031) gene has been disrupted with a <i>hyg</i> cassette	This study
<i>ung</i> [−]	<i>M. smegmatis</i> mc ² 155 in which the <i>ung</i> gene has been disrupted with a <i>kan</i> cassette	Venkatesh <i>et al.</i> (2003)
<i>ung</i> [−] / <i>udgB</i> [−]	<i>M. smegmatis</i> mc ² 155 in which both <i>udgB</i> and <i>ung</i> have been disrupted with a <i>hyg</i> cassette and a <i>kan</i> cassette, respectively	This study
<i>ung</i> [−] / <i>udgB</i> [−] (L5att:: <i>udgB</i>)	<i>M. smegmatis</i> mc ² 155 <i>ung</i> [−] / <i>udgB</i> [−] strain harbouring pMV361 <i>Msm-udgB</i> (Gen ^R) at the L5 <i>att</i> site in the chromosome	This study

column equilibrated with buffer A (Bio-Rad) and eluted with a 30 ml linear gradient of 0.1–1 M NaCl in buffer A. The fractions containing UdgB were checked by SDS-PAGE as well as by UdgB activity assays using GU9 substrate. Fractions were pooled and refractionated on the Ni-NTA column. The fractions containing the protein purified to near homogeneity were pooled, dialysed against buffer B [20 mM HEPES, pH 7.5, 50 % glycerol (v/v), 100 mM NaCl and 10 mM β -mercaptoethanol], quantified using BSA as a standard and stored at -20°C . Uracil excision assays were performed as described previously (Srinath *et al.*, 2007).

Disruption of the *udgB* gene in *M. smegmatis* mc²155 and its *ung*[−] derivative. The upstream (left) flank of the *udgB* gene was PCR-amplified using primers MsmUdgB-LF-Fp, which contains an *StuI* site and anneals ~103 bp upstream of the UdgB start codon, and MsmUdgB-LF-Rp, which contains a *KpnI* site and anneals ~416 bp downstream of the start codon. Similarly, the downstream (right) flank of the *udgB* gene was amplified using a forward primer MsmUdgB-RF-Fp, which contains a *HindIII* site and anneals ~210 bp upstream of the *udgB* stop codon, and a reverse primer MsmUdgB-RF-Rp, which contains a *BglII* site and anneals ~291 bp downstream of the *udgB* stop codon. In both the cases, PCR was carried out in 50 μl volumes containing 2.0 U *Taq* DNA polymerase, 300 ng *M. smegmatis* mc²155 genomic DNA, 20 pmol of each primer pair, 2 % DMSO, *Taq* reaction buffer and 200 μM dNTPs. After initial denaturation for 4 min at 94°C , 30 cycles of incubations were done at 94°C for 1 min, 55°C for 30 s and 70°C for 45 s, followed by a final extension step at 70°C for 10 min. The left flank amplicon of 519 bp was cloned into the pGEM-T Easy vector and digested with *KpnI* and *StuI* for its further subcloning into the pYUB854 vector (Bardarov *et al.*, 2002) and to generate an intermediate clone, pYUB-UdgB-LF. The right flank amplicon of 501 bp was also cloned into the pGEM-T Easy vector and released with *HindIII* and *BglII* for its further subcloning into the same sites of pYUB-UdgB-LF to generate pYUBMsm-*udgB*::*hyg*. pYUBMsm-*udgB*::*hyg* was digested with *NofI* and *SpeI*, and a ~3.2 kb fragment (*udgB*::*hyg*) was mobilized into similarly digested pPR27 (Pelicic *et al.*, 1997) to generate pPRMsm-*udgB*::*hyg*, which was introduced into *M. smegmatis* mc²155 or its *ung*[−] derivative (Venkatesh *et al.*, 2003) by electroporation to generate the single (*udgB*[−]) or double (*ung*[−]/*udgB*[−]) knockout strains using a protocol described previously (Pelicic *et al.*, 1997; Seshadri *et al.*, 2009).

Generation of the *udgB* plasmid (Gen^R) to complement *ung*[−]/*udgB*[−] *M. smegmatis* mc²155. The DNA oligomers MsmUdgB-PFp (which anneals ~180 bp upstream of the start codon) and MsmUdgB-Rp (which anneals ~110 bp downstream of the stop codon) were used to PCR-amplify MSEG_0531 from *M. smegmatis* mc²155 genomic DNA (300 ng) using 1 U DyNAzyme EXT (Finnzyme) in a 25 μl reaction. The PCR conditions included an initial denaturation at 94°C for 4 min, followed by 30 cycles of incubation at 94°C for 1 min, 58°C for 50 s and 72°C for 1 min 30 s, and a final extension at 72°C for 10 min. The amplicon (~1.1 kb) was gel-purified and cloned into pJET1.2 to generate pJET-UdgB. pJET-UdgB was digested with *HindIII* and a fragment of ~1.45 kb containing *udgB* was cloned into a derivative of pMV361 to generate pMV361*udgB*. Subsequently, the gentamicin cassette (Gen^R) from pPR27 (released by *Bam*HI digestion, blunt-ended and digested with *HindIII*) was subcloned between the *Ecl*136II and *HindIII* sites of pMV361*udgB* to replace its Hyg^R marker with the Gen^R marker. pMV361Msm-*udgB* (Gen^R) was introduced into *M. smegmatis* *ung*[−]/*udgB*[−] by electroporation for its ectopic integration into the chromosome at the L5 *att* site to generate the *ung*[−]/*udgB*[−] (L5 *att*::*udgB*) strain.

Southern blot analysis. Genomic DNAs (~2.5 μg) were digested separately with an excess (20 U) of *Bam*HI (MBI Fermentas) and *Pst*II

(Promega) for 20 h, resolved on a 0.7 % agarose gel using Tris-borate-EDTA (TBE), transferred (Reed & Mann, 1985) to a 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 MsmUdgB-LF-Fp and MsmUdgB-RF-Rp (Sambrook *et al.*, 1989). The PCR consisted of ~300 ng template DNA, 20 pmol each primer, 350 μM dATPs, dGTPs and dTTPs, 30 μCi (1110 kBq) [α -³²P]dCTP, ~2 U DyNAzyme II (Finnzyme), 1 $\times$ reaction buffer (Finnzyme) and 2 % DMSO. PCR conditions were as follows: initial denaturation at 94°C for 4 min, 30 cycles of 94°C for 1 min, 55°C for 30 s and 72°C for 2.5 min, and a final extension at 72°C for 10 min.

Immunoblotting of cell extracts from various *M. smegmatis* strains. Cell-free lysates (50 μg total protein) of the various *M. smegmatis* strains were separated on SDS-PAGE gels (15 %) and transferred onto a PVDF membrane (Hybond-P, Amersham Biosciences) using a semi-dry transfer apparatus (Bio-Rad). The membranes were blocked using 0.1 % Tween 20 and 5 % non-fat dried milk in Tris-buffered saline (TBS), and treated with a 1:2000 dilution of rabbit antiserum containing polyclonal antibodies against *M. tuberculosis* UdgB (T. Srinath & U. Varshney, unpublished results) to bind to MsmUdgB, which was then detected using alkaline phosphatase-conjugated goat anti-rabbit IgG with the substrates 5-bromo-4-chloro-3-indolyl phosphate and *p*-nitrotertrazolium blue chloride (BCIP-NBT).

Effect of hydrogen peroxide on bacterial growth. 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 with 0.5 % (w/v) BSA, supplemented with 0, 0.5, 1.0 or 1.5 mM H₂O₂ (Calbiochem), seeded in the microtitre wells of honeycomb plates and incubated in a Bioscreen C kinetic growth reader at 37°C with shaking at maximum amplitude. Growth measurements (OD₆₀₀) were performed at 3 h intervals.

Effect of acidified sodium nitrite. The effect of acidified sodium nitrite on growth was determined in a manner similar to that of H₂O₂ except that the saturated cultures of *M. smegmatis* strains were diluted in LBT (with 0.5 % BSA, and adjusted to pH 5.5) and supplemented with 0, 0.5, 1.5 or 2.5 mM freshly prepared sodium nitrite (Merck).

Determination of mutation rates. Mutation rates were determined exactly as described previously (Kurthkoti *et al.*, 2008).

Analysis of the rifampicin resistance determining region (RRDR). *M. smegmatis* cultures grown for 48 h were plated on LBT agar containing rifampicin (50 $\mu\text{g ml}^{-1}$). The isolated colonies were suspended in 20 μl water, incubated at 90°C for 5 min and centrifuged at 13 000 r.p.m. for 5 min in a table-top centrifuge, and the supernatant was used as template to PCR-amplify the RRDR using 306-*rpoB*-Fp and 306-*rpoB*-Rp primers (Kurthkoti *et al.*, 2010). The PCR products were analysed on a 1 % agarose gel and eluted, and sent for sequencing by MacroGen, using Msm-*rpoB*-seq-Fp.

RESULTS

Identification of MSMEG_5031 as MsmUdgB, and its overproduction and purification from *E. coli*

To overproduce and purify MsmUdgB, we first identified MSMEG_5031 as the homologue of *M. tuberculosis* UdgB by computational methods (Fig. 1). The amino acid sequence analysis revealed that the MSMEG_5031 amino

Fig. 1. Alignment of UdgB (Rv 1259) from *M. tuberculosis* with its homologue MSMEG_5031 from *M. smegmatis*. Sequence alignment was performed with CLUSTAL W and visualized with Boxshade 3.2.1. Conserved motifs A and B are indicated. Dark and mid-grey shading of the sequences indicates identical and similar amino acids, respectively.

acid sequence is ~59% identical to *MtuUdgB*. Furthermore, both the motifs A and B represented by GLAPA and HPSQQN, respectively, are also highly conserved, suggesting MSMEG_5031 to be a family 5 UDG. To determine the biochemical properties of MSMEG_5031, we generated the pTrcMsmUdgB expression construct (which contained a 20 aa N-terminal pre-sequence harbouring the hexahistidine sequence), and to avoid the contaminating activity of Ung, *MsmUdgB* was purified from *E. coli* BW310, an *ung*⁻ strain, using multiple chromatography steps (Methods). Also, as has been shown previously that the presence of the same pre-sequence in the N-terminal region of *MtuUdgB* does not interfere with its activity (Srinath *et al.*, 2007), attempts were not made to cleave the pre-sequence from the purified protein for activity assays.

***M. smegmatis* UdgB is a dsDNA-specific, Ugi-insensitive and thermo-tolerant UDG**

To establish that the MSMEG_5031-encoded protein is indeed *MsmUdgB*, we analysed the key biochemical properties of the purified protein. As shown in Fig. 2(a), the purified protein (MSMEG_5031) excises uracil from GU9, a dsDNA substrate (S) containing uracil in a G:U pair at position 9 from the 5' end, and generates a product (P); and this activity is insensitive to the presence of Ugi, a highly specific *Bacillus subtilis* phage PBS1-encoded proteinaceous inhibitor (Acharya *et al.*, 2003) of the family 1 UDGs (lanes 3–5). The MSMEG_5031-encoded protein did not show uracil excision activity on SSU9, an ssDNA substrate containing uracil within the same sequence context as dsDNA (lanes 1 and 2). Furthermore, when the purified protein was preheated at 37, 40, 45, 50, 55 and 60 °C for 10 min, and then supplemented with the substrate for the assays, the activity declined only when the reaction was carried out at temperatures beyond 50 °C (Fig. 2b, compare lanes 2–7 with the no-protein control in lane 1). In fact, the optimal activity of the protein occurred at 50 °C, indicating it to be a heat-tolerant UDG. Taken together, these properties of the MSMEG_5031-encoded

protein confirm that it is a true homologue of *MtuUdgB*, and like *MtuUdgB*, represents the family 5 UDG (*MsmUdgB*) of *M. smegmatis*.

Generation and characterization of *udgB*⁻ and *ung*⁻/*udgB*⁻ knockouts in *M. smegmatis*

To generate *udgB* gene knockout in *M. smegmatis*, the region between nucleotides 5 127 192 and 5 126 963 of the genome (internal 229 bp of the *udgB* gene) was replaced by the ~1.3 kb *hyg* cassette (Fig. 3a) by introduction of pPRMsm-*udgB*::*hyg*, a derivative of pPR27 (Pelicic *et al.*, 1997), into *M. smegmatis* mc²155 (wild-type) and its *ung*⁻ derivative (Venkatesh *et al.*, 2003). The putative knockout strains, initially identified by colony PCR screening (results not shown), were processed for confirmation of the *udgB* gene disruption by genomic blot analysis (Fig. 3b). As expected from Fig. 3(a), hybridizing bands of ~1.3 and ~3.1 kb for the wild-type (*udgB*) and the disrupted (*udgB*::*hyg*) loci, respectively, in the *Bam*HI digest (Fig. 3b, lanes 1 and 2), and bands of ~3.9 kb (*udgB*) and ~3.0 plus ~2.6 kb (*udgB*::*hyg*) in the *Pst*I digest (lanes 3 and 4) of the genomic DNA of the respective strains were detected, confirming the generation of the desired *udgB* gene knockout. Similarly, the authenticity of the *udgB* gene knockout in the *ung*⁻ *M. smegmatis* was confirmed by *Bam*HI digestion of the genomic DNA of the relevant strains (Fig. 3b, lanes 5 and 6).

Further confirmation of UdgB depletion in the *udgB* knockout strains was carried out by immunoblot analysis using polyclonal antibodies against *MtuUdgB* which cross-reacted with *MsmUdgB* (Fig. 3c). As expected, while the wild-type and the *ung*⁻ strains of *M. smegmatis* showed the presence of *MsmUdgB* (lanes 2, 4 and 6), the *udgB*⁻ strains showed no bands corresponding to UdgB (lanes 3, 5 and 7). However, the *ung*⁻/*udgB*⁻ strain, complemented with an ectopic copy of the *udgB* gene in the L5 *att* site, showed the presence of UdgB (lane 8). Also, the UDG assays (using GU9) of the cell-free lysates (Fig. 3d) revealed that the *udgB*⁻ strain, as expected, possessed only the Ugi-sensitive

Fig. 2. (a) UDG activity assay with purified UdgB. DNA oligomers (5 pmol, containing 10 000 c.p.m. of the same substrate 5' 32 P-labelled) were used. Standard assays with *MsmUdgB* (1 μg) were carried out in 10 μl reactions in UdgB buffer [20 mM Tris/HCl (pH 7.5), 100 mM NaCl, 10% (v/v) glycerol and 2 mM β-mercaptoethanol] for 15 min at 37 °C. The reactions were stopped by addition of 10 μl 0.2 M NaOH and heating at 90 °C for 10 min, mixed with 10 μl sequencing dye (80% formamide, 0.05% each of bromophenol blue and xylene cyanol FF, 10 mM NaOH, 2 mM Na₂EDTA) and boiled for 5 min, and 15 μl aliquots were subjected to electrophoresis on 15% polyacrylamide (19:1) 8 M urea gels and analysed on an FLA2000 BioImage Analyser (Fuji). (b) Heat stability of *MsmUdgB*. Reactions (8 μl) containing UdgB protein (1 μg, lanes 2–7) in 1.25× reaction buffer were heated for 10 min at the temperatures indicated at the top of each lane, supplemented with GU9 (5 pmol, 10 000 c.p.m. in 2 μl) to initiate the reactions, and incubated for a further 10 min at the same temperature. All reactions were then stopped with NaOH and analysed as above. Lane 1 is a substrate-only control treated at 60 °C. Substrate and product bands are indicated by S and P, respectively.

UDG (family 1 UDG, Ung) activity (compare lanes 4 and 5) and that the double-knockout (*ung*[−]/*udgB*[−]) strain possessed no detectable UDG activity (lanes 8 and 9). The control *ung*[−] strain possessed UDG (UdgB) activity which was insensitive to Ugi (lanes 6 and 7), and the wild-type strain possessed UDG activity, a major part of which was due to the family 1 UDG (Ung) and was Ugi-sensitive (lanes 2 and 3).

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

To study the effect of oxidative stress on the UdgB-deficient strains, we studied the growth properties of the strains in the presence of hydrogen peroxide (Fig. 4). Interestingly, in the absence of any added hydrogen peroxide (Fig. 4a), while both the single knockouts (*ung*[−] or *udgB*[−], curves 3 and 2), grew the same as a Kan^R derivative of the wild-type strain [wild-type (Kan), curve 1], the double-knockout strain showed a distinct increase in the lag phase (curve 4). This growth defect in the double-knockout strain was rescued by ectopic insertion of the *udgB* gene at the L5 *att* site in the genome (curve 5), suggesting that the observed effect was specific to UdgB deficiency (under the deficiency of Ung) and not an indirect consequence of genomic disruption at the *udgB* locus. Using a similar complementation analysis, we have previously (Kurthkoti *et al.*, 2008) shown that the effects of *ung* deficiency in the strains are specific to Ung deficiency. Also, as observed in our previous study, the Ung-deficient strain showed an increase in the lag phase when 1.5 mM

H₂O₂ was added to the medium (Fig. 4d, curve 3). Under the same conditions, the *udgB*[−] strain grew as well as the wild-type parent (curves 1 and 2). However, at 1.5 mM H₂O₂, growth of the double-knockout strain was the most impaired. In fact, this was the only strain that revealed an effect even at the lower concentrations of H₂O₂ (0.5 and 1.0 mM, Fig. 4b, c). These observations show that UdgB deficiency leads to a synergistic effect with Ung deficiency. As a control, the growth of the double-knockout strain (*udgB*[−]/*ung*[−]) complemented with an ectopic copy of *udgB* in the L5 *att* site of the chromosome, as expected, was restored to that seen for the *ung*[−] strain (Fig. 4d, curves 3 and 5).

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

We studied the effect of acidified sodium nitrite, which results in the generation of RNI (Fig. 5). As a decrease in the pH of the medium increases the stability of RNI, the experiments necessitated the use of a lower pH for the medium. While the low pH of 5.5 had no effect on the growth of the single knockouts (*ung*[−] or *udgB*[−]), the growth of the double knockout (*ung*[−]/*udgB*[−]) was severely affected (Fig. 5a, compare curve 4 with curves 1–3 and 5), and when the medium was supplemented with sodium nitrite, the strain ceased to grow (Fig. 5b–d curves 4). Interestingly, however, we showed that with an increasing concentration of sodium nitrite, growth of both the single knockouts was also somewhat compromised. However, the effect on the *ung*[−] strain was more than that on the *udgB*[−] strain (Fig. 5b–d curves 3 and 2), and the growth of the

Fig. 3. Generation and characterization of *M. smegmatis* *udgB*⁻ (*udgB::hyg*) strains. (a) Schematic representation of the expected sizes of the fragments in genomic blots from the wild-type and the disrupted *udgB::hyg* alleles of *udgB*. The location of the *udgB*-specific probe used in the genomic blot is indicated by a dotted line above the *udgB* gene. (b) Genomic blot of *M. smegmatis* strains. Lanes: 1 and 3, *M. smegmatis* (wild-type); 2 and 4, *M. smegmatis* *udgB::hyg*; 5, *M. smegmatis* *ung::kan*; 6, *M. smegmatis* *ung::kan, udgB::hyg*. Sizes of DNA bands (in kb) are as indicated. (c) Immunoblot analysis of the cell-free lysates from *udgB*⁻, *ung*⁻, *ung*⁻/*udgB*⁻ and wild-type (Kan) strains were probed with polyclonal antibodies against *MtuUdgB*. Cell-free lysates (50 µg total protein) were loaded on 15 % SDS-PAGE, probed with primary (1 : 2000 dilution) and secondary (1 : 3000 dilution) antibodies, and detected with BCIP-NBT. (d) UdgB activity assays. DNA oligomers (10 000 c.p.m.) were used. Assays with cell-free lysates of the various *M. smegmatis* strains (60 µg) were carried out in 20 µl reactions in UdgB buffer for 15 min at 37 °C. The reactions were stopped by addition of 10 µl 0.2 M NaOH and heating at 90 °C for 10 min, mixed with 10 µl sequencing dye and boiled for 5 min, and 20 µl aliquots were subjected to electrophoresis on 15 % polyacrylamide (19 : 1) 8 M urea gels and analysed using an FLA2000. The bands corresponding to product and substrate are indicated by P and S, respectively.

udgB⁻/*ung*⁻ strain complemented with an ectopic copy of *udgB* at the L5 *att* site of the chromosome was substantially restored to that of the *ung*⁻ strain (Fig. 5b–d, curves 3–5).

Analysis of mutation rate

The mutation rates in an organism are indicative of the DNA repair capacity that processes spontaneously arising damage in the genome. To investigate the contribution of *udgB* to the overall DNA repair process and its possible relationship with *ung*, we determined the mutation rates of *M. smegmatis* mc²155 and its derivatives by scoring for

spontaneously arising rifampicin resistance (Rif^R) in the culture. As shown in Table 2, the deficiency of Ung in *M. smegmatis* mc²155 (*ung*⁻) resulted in an ~8.4-fold increase in the mutation rate (relative to the wild-type), which is in agreement with our previous observations (Kurthkoti *et al.*, 2008). The deficiency of UdgB in the *udgB*⁻ strain resulted in an ~2.1-fold increase in the mutation rate compared with the parental background, suggesting a smaller contribution of UdgB to the overall DNA repair process. However, the mutation rate of the double-knockout (*udgB*⁻/*ung*⁻) strain showed a synergistic increase in the mutation rate to ~19.6-fold compared with the parental strain.

Fig. 4. Effect of H₂O₂ on growth. Saturated cultures of various strains of *M. smegmatis* [wild-type (Kan), *udgB*⁻, *ung*⁻, *ung*⁻/*udgB*⁻ and *ung*⁻/*udgB*⁻ (L5att::*udgB*)] were diluted 100-fold in LBT with 0.5% (w/v) BSA. H₂O₂ was either not added (a) or added at 0.5 mM (b), 1.0 mM (c) and 1.5 mM (d) concentrations. Cultures were grown at 37 °C in a Bioscreen C kinetic growth reader using honeycomb plates. The culture growth was monitored by measuring OD₆₀₀ at regular intervals. Growth curves were prepared from three independent colonies for each strain, and the mean ± SD was plotted as shown.

Mutation spectrum of the various strains

A variety of mutations in the RRDR of the *rpoB* gene are responsible for the origin of Rif^R in the culture. Hence, to understand the underlying mechanism of the spontaneously arising mutations due to UdgB deficiency in the wild-type and *ung*⁻ backgrounds (Table 3), we isolated the RRDR sequences of the Rif^R colonies by PCR and determined their nucleotide sequences. The nature of the nucleotide changes and the frequency of their occurrence in the parent strain and its three derivatives (*ung*⁻, *udgB*⁻ and *ung*⁻/*udgB*⁻) are summarized in Table 3. Consistent with the earlier observations, CG to TA mutations (52%) made a major contribution to the acquisition of Rif^R in the wild-type background, and the contribution of these mutations in the *ung*⁻ background was almost complete (90.9%). On the other hand, in the *udgB*⁻ strain, there was a small increase in the accumulation of AT to GC mutations (45.8% compared with 34% in the wild-type). Interestingly, the level of CG to TA mutations remained nearly the same as in the wild-type background, suggesting that although UdgB contributes to uracil repair in *E. coli* (Srinath *et al.*, 2007), this contribution must be small and secondary to its other repair activities. The AT to GC

mutations could, for example, arise due to lack of Hx (deamination product of A) repair (Srinath *et al.*, 2007). Not unexpectedly, however, due to the domination of the mutation spectrum by a huge background of CG to TA mutations (*ung*⁻ effect), the effect of UdgB in the double knockout could not be recognized.

DISCUSSION

Pathogenic mycobacteria, the causative agents of tuberculosis and leprosy in humans, represent one of the most successful groups of pathogenic micro-organisms. They multiply inside the host macrophages, where they are subjected to RNI and ROS, and because of their G + C-rich genomes, it is expected that cytosine deamination would be one of the major types of damage to their genomes. Hence, these bacteria must possess robust mechanisms to protect and maintain their genetic blueprint. We have shown that Ung, a family 1 UDG, plays an important role in mutation prevention and the endurance of *M. smegmatis* in macrophages *in vitro* (Venkatesh *et al.*, 2003). Furthermore, by using a library of *M. tuberculosis* strains containing randomly inserted transposons in their gen-

Fig. 5. Effect of acidified NaNO_2 on growth. Saturated cultures of various strains of *M. smegmatis* [wild-type (Kan), *udgB*⁻, *ung*⁻, *ung*⁻*udgB*⁻ and *ung*⁻*udgB*⁻ (L5att::*udgB*)] were diluted 100-fold in LBT (pH 5.5) with 0.5% (w/v) BSA, containing no NaNO_2 (a), 0.5 mM NaNO_2 (b), 1.5 mM NaNO_2 (c) or 2.5 mM NaNO_2 (d), and grown at 37 °C in a Bioscreen C kinetic growth reader using honeycomb plates. The culture growth was monitored by measuring OD₆₀₀ at regular intervals. Growth curves were prepared from three independent colonies for each strain, and the mean $\pm$ SD was plotted against time.

omes, it has been shown that Ung is crucial to *M. tuberculosis* for survival in the host (Sasseti & Rubin, 2003). These observations highlight the significance of UDGs in the pathogenesis of mycobacteria and corroborate the use of *M. smegmatis* in assessing the physiological significance of DNA repair proteins.

Given that Ung is crucial for its survival in the host, it is not surprising that *M. tuberculosis* possesses another uracil excision activity, UdgB, which is a family 5 dsDNA-specific UDG. *MtuUdgB* can excise not only uracil but also Hx and ethenocytosine (Srinath *et al.*, 2007). While, using *E. coli* as a host, we have demonstrated that *MtuUdgB* can function

Table 2. Effect of deficiency in DNA repair enzymes on mutation rate

Strain	Total number of viable bacteria plated ($\times 10^9$)*	Mutation rate ($\times 10^{-10}$)†	Fold increase
<i>M. smegmatis</i>	8.6 ± 0.91	1.41	1.0
<i>M. smegmatis ung</i> ⁻	11.0 ± 0.54	11.72	8.4
<i>M. smegmatis udgB</i> ⁻	8.6 ± 1.8	2.89	2.1
<i>M. smegmatis ung</i> ⁻ / <i>udgB</i> ⁻	8.4 ± 0.52	27.57	19.6

*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 $a = 2 \times \log_2[(M_t/N_t) - (M_0/N_0)]/n$, where a =mutation rate, n =number of generations, M_t =number of Rif^R colonies obtained from the 6 day culture (Methods), N_t =number of bacteria in the 6 day culture, M_0 =number of Rif^R colonies in the starter diluted culture used to make the 6 day culture, and N_0 =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 nil, the simplified equation $a = 2 \times \log_2(M_t/N_t)/n$ was used to calculate mutation rates. Mutation rates were calculated for all 12 replicates of the 6 day cultures, and the average mutation rates shown were calculated from 10 replicates, eliminating the two with the highest and the lowest rates.

Table 3. Spectrum of mutations in the RRDR in Rif^R isolates of *M. smegmatis* strains

Mutations detected	Strain			
	<i>M. smegmatis</i> wild-type*	<i>M. smegmatis</i> <i>ung</i> ⁻ †	<i>M. smegmatis</i> <i>udgB</i> ⁻ ‡	<i>M. smegmatis</i> <i>ung</i> ⁻ / <i>udgB</i> ⁻ §
CG→TA	52 % (30/58)	90.9 % (40/44)	45.8 % (22/48)	91.6 % (44/48)
AT→GC	34 % (20/58)	4.5 % (2/44)	45.8 % (22/48)	4.2 % (2/48)
AT→TA	5 % (3/58)	4.5 % (2/44)	0	4.2 % (2/48)
AT→CG	2 % (1/58)	0	6.2 % (3/48)	0
GC→CG	7 % (4/58)	0	2 % (1/48)	0
No mutation in RRDR	21/79	8/52	19/67	12/60

*A total of 79 samples were sequenced, of which 58 showed mutations in the RRDR (reproduced from Kurthkoti *et al.*, 2010).

†A total of 52 samples were sequenced, of which 44 showed mutations in the RRDR.

‡A total of 67 samples were sequenced, of which 48 showed mutations in the RRDR.

§A total of 60 samples were sequenced, of which 48 showed mutations in the RRDR.

||Mutations elsewhere resulted in resistance to rifampicin.

as a UDG *in vivo*, its physiological role in *M. tuberculosis* could not be established because of the unavailability of the *udgB*⁻ strain. However, recently, a homologue of *MtuUdgB* (MSMEG_5031) has been identified in *M. smegmatis*, which provided us with a more tractable genetic system to assess its physiological significance.

In this study, by carrying out biochemical characterization, we have established that MSMEG_5031 (*MsmUdgB*) is a true homologue of *MtuUdgB* (Figs 1 and 2). Furthermore, our studies with the *udgB*⁻ strain of *M. smegmatis* reveal that UdgB deficiency does not significantly affect the growth of the strain in the presence of either H₂O₂ or acidified sodium nitrite under the conditions used. However, when the *udgB*⁻ allele was combined with the *ung*⁻ allele in the same strain of *M. smegmatis*, the effect of Ung deficiency was remarkably enhanced. In fact, the growth of the double-knockout strain (*ung*⁻/*udgB*⁻) was compromised even in the absence of any added DNA-damaging agents (Figs 4 and 5).

Similarly, we observed that a *udgB* knockout in *M. smegmatis* did not lead to a significant increase in mutation rates (approximately twofold over the wild-type). However, its effect was synergistic with that of the *ung*⁻ strain and led to a remarkable enhancement in the mutation rates from approximately eightfold (for the *ung*⁻ strain) to approximately 19-fold (for the *ung*⁻/*udgB*⁻ strain) compared with the wild-type background. Consistent with these observations, while the mutation spectra of the *ung*⁻ and the double-knockout (*ung*⁻/*udgB*⁻) strains were markedly altered from that of the wild-type strain (Table 3), that of the *udgB*⁻ strain had a significant background of the wild-type spectrum but showed a smaller increase in AT to GC mutations, suggesting that UdgB is more important for excision of the adenosine deamination product (Hx), which is consistent with a recent study in *M. smegmatis* (Wanner *et al.*, 2009). However, our findings on the overall

mutation rate of the *udgB*⁻ strain are at a variance with this report, in which the mutation frequency for the UdgB-deficient strain was found to increase ~8.1-fold above the wild-type background and to be very similar to that of the Ung-deficient strain (~7.4-fold). The reasons for this variance remain unclear at present, especially because there is a good agreement between the two studies on the findings with the *ung*⁻ strain. More importantly, however, both studies strongly suggest that UdgB and Ung play synergistic roles in mutation prevention. Finally, taken together with the hypersensitivity of the double-knockout strain (*ung*⁻/*udgB*⁻) to acidified nitrite and oxidative stress, our studies allow us to speculate that generation of a double-knockout (*ung*⁻/*udgB*⁻) strain of *M. tuberculosis* could provide an important attenuated strain for immunological studies.

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