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*.

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,
Here, we identified the MSMEG_5031 from pathogen.

### Table 1. 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 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, 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-hydroxy-methyl-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 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, 1997). GU9, a tetra-loop hairpin oligomer containing uracil in the 5' derivative to study the effects of the two UDGs on mutation rates, mutation spectra and growth under conditions that mimic physiological stresses.

**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 (Rvi1259; Cole et al., 1998). The ORF of MsmUdgB (MSMEG_5031, nucleotide positions 5127697–5126627) was amplified by PCR from *M. smegmatis* mc²155 genomic DNA using a forward MsmUdgB-Fp and a reverse MsmUdgB-Rp primer containing Ndel and HindIII sites, respectively, and DnAzyme 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 Ndel and HindIII, cloned into similarly digested pET14b (Novagen) to generate pETMsmUdgB, 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 pETMsmUdgB with NcoI and HindIII and mobilized into similarly digested pTrc99C (GE Healthcare) to generate pTrcMsmUdgB.

**Overexpression and purification of MsmUdgB, and UDG assays.** Colonies of *E. coli* BW310 (ung⁻) harbouring pTrcMsmUdgB 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 ~0.37 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 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
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 Tris-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 MsuMsdgB-LF-Fp, which contains an StuI site and anneals ~103 bp upstream of the UdgB start codon, and MsuMsdgB-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 MsuMsdgB-RF-Fp, which contains a HindIII site and anneals ~210 bp upstream of the udgB stop codon, and a reverse primer MsuMsdgB-RF-Rp, which contains a BgII 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 BgII for its further subcloning into the same sites of pYUBudgB-LF to generate pYUBmsu-udgB::hyg, pYUBmsu-udgB::hyg was digested with NotI and Spel, and a ~3.2 kb fragment (udgB::hyg) was mobilized into similarly digested pPR27 (Pellicc et al., 1997) to generate pPRMsu-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 (Pellicc et al., 1997; Seshadri et al., 2009).

Generation of the udgB plasmid (Gen⁺) to complement ung⁻/udgB⁻ M. smegmatis mc²155. The DNA oligomers MsuMsdgB-PFp (which anneals ~180 bp upstream of the start codon) and MsuMsdgB-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 for 4 min, followed by 30 cycles of incubation at 94 °C for 1 min, 58 °C for 50 s and 72 °C for 2 min 30 s, and a final extension at 72 °C for 10 min. The amplicon (~1.1 kb) was gel-purified and cloned into pET11.2 to generate pET-UdgB. pET-UdgB was digested with HindIII and a fragment of ~1.45 kb containing udgB was cloned into a derivative of pMV361 to generate pMV361udgB. Subsequently, the gentamicin cassette (Gen⁺) from pPR27 (released by BamHI digestion, blunt-ended and digested with HindIII) was subcloned between the EclII/SalI and HindIII sites of pMV361udgB to replace its HygB marker with the Gen⁺ marker. pMV361Msu-udgB (Gen⁺) 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 BamHI (MBI Fermentas) and PstI (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 (Vaishnokraksha et al., 1997) with radiolabelled probe prepared by PCR using [α-32P]dCTP and primers MsuMsdgB-LF-Fp and MsuMsdgB-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 x 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 MsuMsdgB, which was then detected using alkaline phosphatase-conjugated goat anti-rabbit IgG with the substrates 5-bromo-4-chloro-3-indolyl phosphate and p-nitro-tetrazolium 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 (OD₅₆₀) 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 μg 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 MSEG_5031 as MsuMsdgB, and its overproduction and purification from E. coli

To overproduce and purify MsuMsdgB, we first identified MSEG_5031 as the homologue of M. tuberculosis UdgB by computational methods (Fig. 1). The amino acid sequence analysis revealed that the MSEG_5031 amino
To establish that the MSMEG_5031-encoded protein is insensitive and thermo-tolerant UDG.

M. smegmatis activity assays.

cleave the pre-sequence from the purified protein for purified from avoid the contaminating activity of Ung, sequence harbouring the hexahistidine sequence), and to sidedion construct (which contained a 20 aa N-terminal pre-

together, these properties of the MSMEG_5031-encoded properties of the purified protein. As shown in Fig. 2(a), indeed Msm

the reaction was carried out at temperatures beyond 50 u C, indicating it to be a heat-tolerant UDG. Taken

in acid sequence is ~59% identical to MtuUdgB. Furthermore, both the motifs A and B represented by

GLAPA and HPSQQQN, 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 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 (Achariya 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 ung- and ugdB- knockouts in *M. smegmatis***

To generate *udgB* gene knockout in *M. smegmatis*, the region between nucleotides 512792 and 512963 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 (Pellicic 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*) locus, respectively, in the *BamHI* 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 *PstI* 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 *BamHI* 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, 3 and 4), the *udgB* strains showed no bands corresponding to UdgB (lanes 5, 6 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...
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 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 aerobic (Kan), curves 1–3 and 5).

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udgB<sup>−</sup>/ung<sup>−</sup> strain complemented with an ectopic copy of udgB at the L5 att site of the chromosome was substantially restored to that of the ung<sup>−</sup> 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* mc<sup>2</sup>155 and its derivatives by scoring for spontaneously arising rifampicin resistance (Rif<sup>R</sup>) in the culture. As shown in Table 2, the deficiency of Ung in *M. smegmatis* mc<sup>2</sup>155 (ung<sup>−</sup>) 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<sup>−</sup> 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<sup>−</sup>/ung<sup>−</sup>) strain showed a synergistic increase in the mutation rate to ~19.6-fold compared with the parental strain.

**Fig. 3.** Generation and characterization of *M. smegmatis* udgB<sup>−</sup> (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<sup>−</sup>, ung<sup>−</sup>, ung<sup>−</sup>/udgB<sup>−</sup> 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.
Mutation spectrum of the various strains

A variety of mutations in the RRDR of the rpoB gene are responsible for the origin of RifR in the culture. Hence, to understand the underlying mechanism of the spontaneously arising mutations due to UdgB deficiency in the wild-type and ung2 background (Table 3), we isolated the RRDR sequences of the RifR 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 (ung2, udgB2 and ung2/udgB2) are summarized in Table 3. Consistent with the earlier observations, CG to TA mutations (52%) made a major contribution to the acquisition of RifR in the wild-type background, and the contribution of these mutations in the ung2 background was almost complete (90.9%). On the other hand, in the udgB2 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 (ung2 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-
omes, it has been shown that Ung is crucial to *M. tuberculosis* for survival in the host (Sassetti & 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. *Mtu UdgB* 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 *Mtu UdgB* can function

**Fig. 5.** Effect of acidified NaNO₂ 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 NaNO₂ (a), 0.5 mM NaNO₂ (b), 1.5 mM NaNO₂ (c) or 2.5 mM NaNO₂ (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 ± SD was plotted against time.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Total number of viable bacteria plated (×10⁹)*</th>
<th>Mutation rate (×10⁻⁶)†</th>
<th>Fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. smegmatis</em></td>
<td>8.6 ± 0.91</td>
<td>1.41</td>
<td>1.0</td>
</tr>
<tr>
<td><em>M. smegmatis ung</em>⁻</td>
<td>11.0 ± 0.54</td>
<td>11.72</td>
<td>8.4</td>
</tr>
<tr>
<td><em>M. smegmatis udgB</em>⁻</td>
<td>8.6 ± 1.8</td>
<td>2.89</td>
<td>2.1</td>
</tr>
<tr>
<td><em>M. smegmatis ung/udgB</em>⁻</td>
<td>8.4 ± 0.52</td>
<td>27.57</td>
<td>19.6</td>
</tr>
</tbody>
</table>

*Mean ± 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 \( a = 2 \times \log([M/N]_t - [M/N]_0)/n \), where \( a \) = mutation rate, \( n \) = number of generations, \( M \) = number of Rif⁺ colonies obtained from the 6 day culture (Methods), \( N \) = number of bacteria in the 6 day culture, \( M_0 \) = number of Rif⁺ 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 \( a = 2 \times \log([M/N]_t)/n \) 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.
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 *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 but did not lead to a significant increase in mutation rates (approximately twofold over the wild-type).

**Table 3. Spectrum of mutations in the RRDR in RifR isolates of *M. smegmatis* strains**

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

*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 60 samples were sequenced, of which 48 showed mutations in the RRDR.§A total of 67 samples were sequenced, of which 44 showed mutations in the RRDR.‖Mutations elsewhere resulted in resistance to rifampicin.

The 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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