Substrate specificities and functional characterization of a thermo-tolerant uracil DNA glycosylase (UdgB) from Mycobacterium tuberculosis

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ABSTRACT

Uracil DNA glycosylases (UDGs) excise uracil from DNA and initiate the base (uracil) excision repair pathway. Ung, a highly conserved protein, is the only UDG characterized so far in mycobacteria. Here, we show that Rv1259 from Mycobacterium tuberculosis codes for a double-stranded DNA (dsDNA) specific UDG (MtuUdgB). MtuUdgB is thermo-tolerant, contains Fe–S cluster and, in addition to uracil, it excises ethenocytosine and hypoxanthine from dsDNA. MtuUdgB is product inhibited by AP-site containing dsDNA but not by uracil. While MtuUdgB excises uracil present as a single-nucleotide bulge in dsDNA, it is insensitive to inhibition by dsDNA containing AP-site in the bulge. Interestingly, in the presence of cellular factors, the uracil excision activity of MtuUdgB is enhanced, and when introduced into E. coli (ung−), it rescues its mutator phenotype and prevents C to T mutations in DNA. Novel features of the mechanism of action of MtuUdgB and the physiological significance of the family 5 UDG in mycobacteria have been discussed.

1. Introduction

Deamination of the exocyclic amino group of cytosine residues results in occurrence of uracils in DNA as U:G base pairs. The conversion of C to U is one of the most common damages in DNA that occurs even under normal physiological conditions and can be exacerbated by environmental mutagens [1]. A deficiency in repairing this damage prior to DNA replication results in C:G to T:A mutations in the progeny. Occasional incorporation of dUMP during replication also results in occurrence of uracils in DNA as U:A base pairs. While the presence of such uracils in DNA does not change the genetic information, their transient presence in the genome may interfere with the interaction of sequence specific DNA binding proteins [2,3]. Thus, to maintain genomic integrity, a class of enzymes known as uracil DNA glycosylases (UDGs) excise uracils from DNA and initiate the base excision repair pathway [4].

Of the known UDGs, Ung proteins (family 1 UDGs) are the most conserved, highly efficient and by far the best characterized UDGs. The family 1 UDGs excise uracils from both the single-stranded (ss) and double-stranded (ds) DNAs [5]. Structural and functional characterization of the family 1 UDGs has established the importance of two highly conserved amino acid sequence motifs, A and B (GQDPY and HPSPLS, respectively) in the cleavage of the N-glycosidic bond between uracil and the deoxyribose sugar [6,7]. The dsUDGs (also known as MUG or DUG, family 2) which excise uracil or thymine from G:U/T mismatches in dsDNA, possess GINPG and MPSSSAR as motifs A and B, respectively [8]. The ssDNA selective monofunctional UDGs (SMUG, family 3) possess GMNPG and HPSPRNP as motifs A and B, respectively [9]. SMUG also acts on...
dsDNA but requires AP endonuclease for the product release. Recently, two more UDGs, UdgA (family 4) containing GEAPG and HPAAVLR, and UdgB (family 5) possessing GLAPA and HPSPLNV sequences as motifs A and B, respectively have been characterized from *Thermus thermophilus* and *Pyrobaculum aerophilum* [10–13].

Three-dimensional structures of none of the family 5 UDGs, to which UdgB belongs, have been determined. The structures of the remaining UDGs [9,10,14] show that despite the lack of a significant overall sequence homology, the geometry of the active site pockets of UDGs is conserved, and their catalytic efficiencies may depend on the extent of attenuation of the active site motifs A and B. UDGs known so far are monofunctional and the sugar residues resulting in an AP-site [15]. However, the bifunctional DNA glycosylases react further to eliminate the AP-deoxyribose moiety in a multi-step reaction [16]. Importantly, of the known UDGs, only the family 1 UDGs (Ung proteins) are inhibited by *Bacillus subtilis* phage PBS-1, PBS-2 encoded, Ugi which forms a tight complex with them in 1:1 molar stoichiometry [17].

In addition to uracil, PaeUdgB excises 5-hydroxymethyluracil, ethenocytosine, 5-fluorouracil and hypoxanthine from dsDNA [11]. A computational analysis [11] revealed that a gene (Ru1259) encoding an ortholog of PaeUdgB is also present in *Mycobacterium tuberculosis*, a mesophile and an important pathogen which infects over a third of the world population and causes one of the most dreaded diseases, tuberculosis, in humans and the livestock. Importantly, mycobacteria deficient in Ung show an increased mutator phenotype and a poor endurance in *ex vivo* and *in vivo* models suggesting that UDG activity is crucial for their growth [18,19]. In *P. aerophilum*, UdgB has been suggested to be a backup UDG [20]. Such a function of UdgB in *M. tuberculosis* could be a crucial contributor to the maintenance of genomic integrity and virulence of this G+C rich bacterium. Here, we report the purification of recombinant MtuUdgB and its biochemical and functional characterization which have provided us with novel insights into the mechanism of action of the family 5 UDGs.

### Table 1 – List of the oligodeoxyribonucleotides

<table>
<thead>
<tr>
<th>S.N.</th>
<th>Name</th>
<th>Sequence (5′–3′)</th>
<th>Size (nucleotide)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GU9</td>
<td>ctaaatgUaggcatgttctgcacattgctact tga</td>
<td>37</td>
<td>DNA hairpin containing G:U (in GU9) and A:U (in AU9) at position 9 in stem.</td>
</tr>
<tr>
<td>2</td>
<td>AU9</td>
<td>ctaaatgUaggcatgttctgcacattgctact tga</td>
<td>37</td>
<td>U in a bulge in stem region of a hairpin.</td>
</tr>
<tr>
<td>3</td>
<td>U-bulge</td>
<td>ttgcctacttagaagtggaggca</td>
<td>26</td>
<td>U at position 9 in single-stranded DNA.</td>
</tr>
<tr>
<td>4</td>
<td>SSU9</td>
<td>ctaaatgUaggcatgttctgcacattgctact tga</td>
<td>24</td>
<td>Complementary to SSU9; 12th base is G.</td>
</tr>
<tr>
<td>5</td>
<td>SSU9-Compl-G</td>
<td>cttctagctgcacattgctactgca</td>
<td>24</td>
<td>Complementary to SSU9; 12th base is C.</td>
</tr>
<tr>
<td>6</td>
<td>SSU9-Compl-C</td>
<td>cttctagctgcacattgctactgca</td>
<td>24</td>
<td>Complementary to SSU9; 12th base is A.</td>
</tr>
<tr>
<td>7</td>
<td>SSU9-Compl-A</td>
<td>cttctagctgcacattgctactgca</td>
<td>24</td>
<td>Complementary to SSU9; 12th base is T.</td>
</tr>
<tr>
<td>8</td>
<td>SSU9-Compl-T</td>
<td>cttctagctgcacattgctactgca</td>
<td>24</td>
<td>14th base is etheno-cytosine.</td>
</tr>
<tr>
<td>9</td>
<td>eC</td>
<td>gactgctgctact(e)gagggaagaaggcc</td>
<td>26</td>
<td>14th base is 5-OH-ctosine.</td>
</tr>
<tr>
<td>10</td>
<td>5-OH-C</td>
<td>gactgctgctact(OH)gagggaagaaggcc</td>
<td>26</td>
<td>14th base is etheno-adenosine.</td>
</tr>
<tr>
<td>11</td>
<td>eA</td>
<td>gactgctgctact(e)agggaagaaggcc</td>
<td>26</td>
<td>14th base is dihydroxy-uridine.</td>
</tr>
<tr>
<td>12</td>
<td>DHU</td>
<td>gactgctgctact(DHU)agggaagaaggcc</td>
<td>26</td>
<td>14th base is hypoxanthine.</td>
</tr>
<tr>
<td>13</td>
<td>Hx</td>
<td>gactgctgctact(Hx)agggaagaaggcc</td>
<td>26</td>
<td>Complementary to oligomers 13–17; nucleotide against the unusual bases in oligomers 13–17 is G, T or C, respectively</td>
</tr>
<tr>
<td>14</td>
<td>Compl-G</td>
<td>ggcacatttcgcttgacacacgctc</td>
<td>26</td>
<td>Forward and reverse primers to amplify.</td>
</tr>
<tr>
<td>15</td>
<td>Compl-T</td>
<td>ggcacatttcgcttgacacacgctc</td>
<td>26</td>
<td>RRDR locus by PCR.</td>
</tr>
<tr>
<td>16</td>
<td>Compl-C</td>
<td>ggcacatttcgcttgacacacgctc</td>
<td>26</td>
<td>Primer used to sequence RRDR.</td>
</tr>
<tr>
<td>17</td>
<td>306-rpo8-Fp</td>
<td>cpgacaccttgccaaacc</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>306-rpo8-Rp</td>
<td>cpgacaccttgccaaacc</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Eco-rpo8-seq-Fp</td>
<td>ggcacatgcttgacacacgctc</td>
<td>18</td>
<td></td>
</tr>
</tbody>
</table>

2. **Materials and methods**

2.1. **Bacterial strains and plasmids**

*E. coli* TG1 was used in recombinant DNA experiments. *E. coli* BW310 (*ung<sup>−</sup>*) and BL21 (DE3) were used for overproduction of recombinant proteins from the pTrc99C and pET14b based expression constructs, respectively. *E. coli* MG1655 and its *ung<sup>−</sup>* derivative containing insertion of kanamycin resistance marker in the active site motif GQDPY (*ung<sup>−</sup>*:kan) (Bharti and Varshney, unpublished) were used to assay for mutator phenotypes.

2.2. **Substrates**

DNA oligomers (Table 1) were obtained from Sigma–Aldrich, India; Ransom Hill Bioscience, USA, and Microsynth, Switzerland. Oligomers were purified using Sep-Pak (C18) cartridges (Waters), quantified by taking absorbance at 260 nm and dissolved in water at 0.1 nmol/ml and 0.1 μCi/ml (where n = number of nucleotides in the oligomer) to obtain a strand concentration of 10 nmol/ml [21].

2.3. **Radiolabeling and generation of double-stranded (ds) DNA oligomers**

DNA oligomers (10 pmol) were 5′-[gamma-<sup>32</sup>P] ATP (6000 Ci/mmol) and T4 polynucelotide kinase and purified on Sephadex G50 minicolumns [22]. The GU9,
a tetra-loop hairpin oligomer containing uracil in the stem region was used as dsDNA substrate. However, when required, other dsDNA substrates were generated by mixing the 5′-32P-end labeled DNA oligomers with three fold molar excess of complementary DNA in 20 mM Tris–HCl (pH 7.5), 10 mM Na2EDTA and 150 mM NaCl, heated at 90 °C for 10 min and allowed to cool to room temperature over a period of 4 h to generate dsDNA oligomers and stored at −20 °C. As UdgB is not inhibited by ssDNA (see Section 3), no further purifications to eliminate unlabelled excess ssDNA oligomers were carried out.

2.4. Cloning of MtuUdgB in pET14b and pTrc99C

The open reading frame (ORF) of MtuUdgB (Ru1259) was amplified by PCR from M. tuberculosis DNA using a forward (5′-GCT GTT TCA TAT GAA TAT CGC GGC T-3′) and a reverse (5′-CCA CAA GCT TCA GCT CAC TCA-3′) primers containing Ndel and HindIII sites, respectively, and Vent DNA polymerase (NEB). The reaction was heated at 94 °C for 4 min followed by 30 cycles of incubations at 94 °C for 1 min, 66 °C for 45 s and 72 °C for 50 s. The PCR product (969 bp) was digested with Ndel and HindIII, cloned into similarly digested pET14b (pETMtuUdgB) and confirmed by DNA sequence analysis. This construct appended a 20 amino acid long (MGSSHHHHHSSGLVPRGSH) pre-sequence to MtuUdgB. Subsequently, MtuUdgB sequence along with the pre-sequence was excised by digestion of pETMtuUdgB with Ncol and HindIII and mobilized into the similarly digested pTrcMtuUdgB to generate pTrcMtuUdgB.

2.5. Over-expression and purification of MtuUdgB

pTrcMtuUdgB was introduced into E. coli BW310 (ung−) by transformation. Cultures (2 l) in LB medium containing ampicillin (100 μg/ml) and ferric chloride (0.01%, w/v) were grown at OD595 of 0.6 at 37 °C from single transformants under shaking, supplemented with 0.5 mM IPTG and the growth continued further for 4 h. Cells were harvested by centrifugation at 4 °C, suspended in buffer 1 [20 mM HEPES (pH 7.5), 10% glycerol (v/v), 500 mM NaCl and 2 mM β-mercaptoethanol], ultrasonicated and subjected to centrifugation for 20 min at 4 °C at 14,000 × g. The supernatant was loaded onto an Ni-NTA column (5 ml, Pharmacia Biotech) pre-equilibrated with buffer 1. The column was washed with 20 ml of buffer 1 containing 10 mM imidazole. MtuUdgB was eluted with a gradient of 10–1000 mM imidazole in buffer 1. The fractions containing MtuUdgB were pooled, dialyzed against buffer 1 and re-fractionated on the Ni-NTA column. The fractions containing the protein purified to near homogeneity were pooled, dialyzed against buffer 2 [20 mM HEPES, pH 7.5, 50% glycerol (v/v), 500 mM NaCl and 10 mM β-mercaptoethanol], quantified [23] using BSA as standard and stored at −20 °C.

2.6. Purification, and subcloning of MtuUng

The pRSETbMtuUng construct [24] encoding MtuUng containing a 40 amino acid long presequence was digested with Ncol and NheI to generate pRSET-11-MtuUng with an 11 amino acids long pre-sequence (MHHHHHHGMAS). The pRSET-11-MtuUng was introduced into E. coli BL21 (DE3) and MtuUng purified essentially as described [24]. Further, the Ndel to HindIII fragment from this expression plasmid was subcloned into pTrc99CNdel between Ndel and HindIII sites to generate a construct for expression of MtuUng in E. coli BW310 (ung−). The pTrc99CNdel was generated by eliminating an internal Ndel site from pTrc99C by its digestion with Ndel followed by end filling and relegation; and then by mutating the Ncol site to an Ndel site.

2.7. Base excision assays using MtuUdgB and MtuUng

Unless stated otherwise, DNA oligomers (5 pmol, containing 10,000 cpm of the same substrate 5′-32P) labeled) were used. Standard assays with MtuUdgB (50 ng, ~1.5 pmol) were carried out in 10 μl reactions in UdgB buffer [20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 10% glycerol and 2 mM β-mercaptoethanol] for 15 min at 37 °C. The assays with MtuUng (30 ng, ~1.2 pmol) were carried out in 20 mM Tris–HCl (pH 7.5). The reactions were stopped by addition of 10 μl of 0.2N 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), boiled for 5 min, and 15 μl aliquots from it were subjected to electrophoresis on 15% polyacrylamide (19:1)-8M urea gels. For the time course experiments, 350 ng (~10.5 pmol) of MtuUdgB was reacted with 35 pmol substrate (containing 70,000 cpm of the same oligomer 5′-32P labeled) in a volume of 70 μl. Samples (10 μl) were drawn at desired time points, and analyzed on 15% polyacrylamide-8M urea gels. Bands corresponding to the substrate and product were quantified by a BioImage Analyzer (FLA2000, Fuji Film, Japan) to calculate the percent product formed (100× counts in product band divided by the total of counts in the product and the substrate bands).

2.8. Assays with immobilized MtuUdgB

Aliquots (3 μl) of the slurry of water washed Ni-NTA beads (Bio-Rad) were spotted on a parafilm sheet kept on a glass plate on ice (seven spots; one for the control and the other six for UdgB reactions). Buffer 2 (3 μl) was added to each spot. After 30 min, 2 μl of the His6-tagged MtuUdgB (3 μg) was added to each of the six spots for UdgB reaction. To the control reaction (−UdgB spot), 2 μl of buffer 2 was added. After 1 h, each spot was supplemented with 4 μl of an RNA, BSA, Ugi mix (50 μg yeast total RNA, 50 μg BSA and 50 ng Ugi in 32 μl solution) to block nonspecific binding sites as well as to inactive contamination from the family 1 UDG (Ung) activity, if any, in the reagents and incubated further for 1 h. The supernatant was removed from each spot and the beads were washed twice with 10 μl aliquots of wash buffer [20 mM Tris–HCl (pH 7.5), 200 mM NaCl, 10% glycerol (v/v), 2 mM β-mercaptoethanol, 50 μg BSA and 5 ng Ugi in 327 μl], and the glass plate was shifted to a 37 °C heating block. The wash buffer was removed and the reactions were started by addition of 15 μl of GU9 [5 pmol, 20,000 cpm in 100 mM NaCl, 10% glycerol (v/v), 2 mM β-mercaptoethanol, 50 μg BSA and 5 ng Ugi] to each bead spot. Supernatants (15 μl) from the series of UdgB bead spots were collected at ~1, 3, 6, 9, 12 and 15 min, respectively. The super-
natant from the control (-UdgB) bead spot was collected at 15 min. The collected supernatants were transferred to eppendorf tubes, spun for 5 s in a microfuge, and 10 µl aliquots taken from the top were retained, mixed with 5 µl of 0.2N NaOH and 10 µl sequencing dye in a new tube and heated at 90 °C for 10 min. On the other hand, immediately after removal of the supernatant, the beads from the series of reaction spots on the parafilm, were mixed with 10 µl of 0.1N NaOH, transferred to eppendorf tubes, mixed with 10 µl sequencing dye and heated at 90 °C for 10 min. The heated samples were analyzed on 15% polyacylamlide-8M urea gels as described above for UdgB reactions.

2.9. Inhibition of MtuUdgB activity by uracil and AP-DNA

MtuUdgB was incubated with varying concentrations of uracil (Sigma–Aldrich) or AP-DNA first at room temperature and then on ice, for 15 min each, and used in standard MtuUdgB assay. The AP-DNA were generated by treating SSU9 and GU9 oligomers (2.5, 5, or 10 pmol) with EcoUng (12 ng) in 4.5 µl reactions consisting of 20 mM Tris–HCl (pH 7.5) at 37 °C for 2 h. To this, 25 ng Ugi (0.5 µl) was added and the reactions incubated at room temperature for 30 min to sequester EcoUng into an inactive EcoUng-Ugi complex. The oligomers so generated were referred to as SSap9 and Gap9, respectively.

2.10. Electrophoretic mobility shift assays (EMSA)

MtuUdgB (120 ng) was incubated with 10,000 cpmp of radiolabeled GU9, Gap9 or SSU9 in EMSA buffer [20 mM Tris–HCl (pH 8.0), 50 mM NaCl, 5% glycerol (v/v), 1 mM β-mercaptoethanol and 50 µg/ml BSA] in 20 µl volumes, for 15 min on ice and resolved on 10% native polyacrylamide (19:1) gels of 0.75 mm thickness and 160 mm length at 150 V in cold room and resolved on 10% native polyacrylamide (19:1) gels of 0.75 mm thickness and 160 mm length at 150 V in cold room. T o this, 25 ng Ugi (0.5 µl) was added and the reactions incubated at room temperature for 30 min to sequester EcoUng into an inactive EcoUng-Ugi complex. The oligomers so generated were referred to as SSap9 and Gap9, respectively.

2.11. Exposure of E. coli harboring pTrc99C, pTrcMtuUdgB or pTrc99MtuUng to acidified nitrite

Isolated colonies of E. coli BW310 (ung⁻) harboring pTrc99C, pTrcMtuUdgB or pTrc99MtuUng were inoculated into 2 ml LB containing ampicillin (100 µg/ml), and grown at 37 °C under shaking to an OD₅₉₅ of 0.6. Pre-warmed LB (20 ml, containing ampicillin and 0.01%, w/v, ferric chloride) was then inoculated with 1% of pre-culture, grown to an OD₅₉₅ of 0.6 under the same conditions, supplemented with 0.5 mM IPTG and the growth of the cultures was continued further for 4 h. The cultures were mixed with tetracycline (7.5 µg/ml) and incubated for 10 min at 37 °C. Cells from the cultures were pelleted at centrifugation at 4 °C, suspended in 20 ml PBS-1 (10 mM phosphate, 150 mM NaCl, pH 5.0), pelleted again by centrifugation and resuspended in PBS-1 (20 ml). Cells (2 ml aliquots) were supplemented with freshly prepared 2M NaNO₂ to final concentrations of 2.5, 5 and 10 mM and shaken for 15 min at room temperature, pelleted by centrifugation at 4 °C for 5 min, washed twice by suspending in 1 ml PBS-2 (10 mM phosphate, 150 mM NaCl, pH 7.5) and taken up in 400 µl of buffer 1, ultrasonicated and centrifuged in a microfuge for 15 min at 4 °C. The supernatant was estimated for total proteins, and assayed for β-lactamase [25].

2.12. Effect of homologous and heterologous cell-free extracts on substrate processing by MtuUdgB

Cell free extracts of M. tuberculosis H37Ra or E. coli BW310 (ung⁻) were prepared as described [24]. Cell-free extracts of M. tuberculosis were supplemented with excess Ugi (20 ng in 2 µg of cell-free extract) and incubated first at room temperature and then on ice for 30 min each to inhibit endogenous Ung activity [24]. Ugi-treated or untreated cell-free extracts (2 µg per reaction) were added to standard MtuUdgB reactions.

2.13. Rescue of mutator phenotype of E. coli (ung⁻) by MtuUdgB

Mutator phenotypes of E. coli MG1655 (ung⁻:kan) harboring pTrc99C, pTrcMtuUng or pTrcMutUdgB were determined by scoring for rifampicin resistant colonies. Briefly, isolated colonies were grown to early log phase (OD₅₉₅ ~0.2–0.3). Cultures were diluted 1000 fold to make seven independent cultures which were grown to late log phase. The cells were harvested and plated on LB agar containing rifampicin (50 µg/ml). Viable counts corresponding to OD₅₉₅ values of each independent culture were determined and calculated from the standard curve plotted between OD₅₉₅ values versus viable counts in the logarithmic range. The mutator phenotype of each strain was calculated by dividing the number of rifampicin resistant colonies by total viable counts of the plated cultures.

2.14. Analysis of rpoB locus of E. coli strain expressing MtuUdgB

The rpoB locus of E. coli was amplified from rifampicin resistant colonies using 306 rpoB-Fp and 306 rpoB-Rp. PCR were performed in 100 µl volumes with 2U of Pfu DNA polymerase, 40 pmol of each primer and 200 µM dNTPs by heating the tube at 94 °C for 4 min followed by 29 cycles of incubations at 94 °C for 1 min, 55 °C for 30 s and 70 °C for 2 min. The PCR products were analyzed by agarose gel electrophoresis and the 306 bp amplicons were recovered from the gel using silica beads (GeneClean II Kit, BIO101) and sequenced using Eco-rpoB-seq-Fp (Macrogen Inc., S. Korea). The spectrum of mutations was determined by aligning the 81 bp rifampicin resistance-determining region (RRDR) of rpoB gene of the RifB colonies with that of the wild type locus.
3. Results

3.1. Identification of a new dsDNA specific, Ugi insensitive and thermo-tolerant uracil DNA glycosylase activity in M. tuberculosis

Analysis of the M. tuberculosis genome revealed that a homolog of the dsUDG (family 2), a dsDNA specific UDG, is missing from this bacterium [26]. However, a more recent computational analysis [11] predicted the presence of a family 5 dsDNA specific UDG, UdgB in mycobacteria. To check for the presence of the dsDNA specific UDG activity in mycobacteria, cell-free extracts were assayed using SSU9 (uracil containing ssDNA) and GU9 (a tetra-loop hairpin dsDNA containing a G:U pair in the middle of the stem). As shown in Fig. 1A, the extracts efficiently process excision of uracil from both the substrates (lanes 2 and 5, respectively). Addition of Ugi, a highly specific inhibitor of Ung (which use both ssDNA and dsDNA as substrates) to the extracts resulted in complete loss of uracil excision from SSU9 (compare lanes 2 and 3). However, under the same conditions, inhibition of uracil excision from GU9 was not complete (compare lanes 5 and 6). As the Ung activity in the M. tuberculosis cell-free extracts is potently inactivated by Ugi [24], a minor activity of GU9 processing in these assays indicates the presence of a previously unrecognized mycobacterial UDG which is insensitive to inhibition by Ugi and acts exclusively on dsDNA. It may be noted that GU9, owing to strong secondary structure (hairpin DNA), migrates as doublet.

MtUdgB represents the only non-family 1 UDG in M. tuberculosis; and the fact that it is also expressed in vivo [19,27,28], the dsDNA specific UDG activity observed in Fig. 1, most likely corresponds to the mycobacterial UdgB.

3.2. Purification of MtUdgB

To further our understanding of UdgB activity in mycobacteria, we cloned UdgB ORF from M. tuberculosis into a derivative of pTrc99C expression vector along with a 20 amino acid long pre-histidine tag. To ensure that MtUdgB preparation was free from any contaminating activity of Ung, MtUdgB was purified from E. coli BW310, an ung− strain (Section 2).

3.3. MtUdgB is heat-tolerant

To examine if the activity of MtUdgB, corresponded to the heat-tolerant activity detected in the total cell extracts, we preheated the purified MtUdgB and MtUng (Section 2) at 40–70 °C for 10 min, and then assayed them for their activities at the temperature they were preheated. As shown in Fig. 2, while the activity of MtUng declined rapidly with increasing temperature, the activity of MtUdgB decreased only when heated beyond 60 °C suggesting the latter to be a heat-tolerant UDG.

3.4. MtUdgB contains Fe–S cluster

TthUdgA, a family 4 UDG, crystal structure has revealed that it contains a Fe–S cluster [10]. Comparison of MtUdgB with TthUdgA (Fig. 3A) shows that the four conserved Cys residues which participate in Fe–S cluster formation in TthUdgA, are also conserved in MtUdgB suggesting that the family 5 proteins may also contain Fe–S cluster. However, the putative Fe–S cluster motif (CysX2CysX100CysX14Cys) contains a stretch.

Fig. 1 – Identification of Ugi insensitive, dsDNA specific and thermo-tolerant UDG activity in M. tuberculosis. (A) Reactions (20 µl) containing ~0.1 pmol (10,000 cpm) of SSU9 (lanes 1–3) or GU9 (lanes 4–6) and 10 µg M. tuberculosis H37Ra cell-free extracts untreated (lanes 2 and 5) or treated with 0.4 µg Ugi (lanes 3 and 6) were carried out for 1 h at 37 °C. Lanes 1 and 4 show substrate alone controls. Reactions were analyzed on 15% polyacrylamide-8M urea gels and visualized by Bioimage Analyzer (FLA2000, Fuji Film). The substrates (SSU9 and GU9), and product (9mer) bands are indicated as S and P. The schematics of reactions with SSU9 and GU9 are shown on the left- and right hand sides, respectively. Star at the 5′-end of the drawings indicates 32P-end labeling. The oligomer GU9, owing to strong secondary structure (hairpin DNA), migrates as doublet. (B) Same as in (A) except that in lanes 3 and 6, cell-free extracts heat-treated at 60 °C resulted in a complete loss of ssDNA processing activity (Ung), it retained a minor activity of processing dsDNA (Fig. 1B, lanes 3 and 6, respectively). Considering that
respectively. Substrate and product bands are indicated with S and P, respectively. Lane 1 is substrate alone control treated at 70 °C. All reactions were then heated for 10 min at temperatures indicated on top of each lane, and supplemented with GU9 (5 pmol, 10,000 cpm in 2 μl) to initiate the reactions, and incubated further for 10 min at the same temperature. All reactions were then treated with NaOH and resolved on 15% polyacrylamide-8M urea gels, visualized and quantified using Bioimage Analyzer. Lane 1 is substrate alone control treated at 70 °C. Substrate and product bands are indicated with S and P, respectively.

of 100 amino acids in MtuUdgB as opposed to an 80 amino acids stretch in TthUdgA. The UV–vis scan of MtuUdgB (Fig. 3B) revealed a peak at ~420 nm, in addition to the expected peaks at 230 and 280 nm signifying the presence of an Fe-S cluster [29] in MtuUdgB. Furthermore, treatment of proteins containing Fe–S cluster is also known to be susceptible to reactive nitrogen species generated by acidified sodium nitrite [30,31]. As shown in Fig. 3C, with an increase in concentration of sodium nitrite used to treat E. coli cells harboring pTrcMtuUdgB, the activity of MtuUdgB in the cell-free extracts decreased (Fig. 3C, compare lanes 11–14). Such a treatment of E. coli cells harboring expression construct for MtuUng, a family 1 UDG, known not to possess Fe–S cluster, did not result in any decrease in its activity (Fig. 3C, lanes 7–10). As a control for plasmid copy number, we assayed for rela-

tivity of MtuUdgB remaining bound to the AP-DNA, we carried out reaction with MtuUdgB in the cell-free extracts extracted from E. coli cells harboring pTrcMtuUdgB. Taken together, with the recognition of a TthUdgA type Fe–S cluster sequence motif in MtuUdgB (Fig. 3A) we suggest that MtuUdgB possesses Fe–S cluster, and that the presence of Fe–S cluster may be a general feature of all the family 5 UDGs.

3.5. Substrate specificity of MtuUdgB and the effect of Ugi on uracil excision

Fig. 4A, shows that MtuUdgB does not excise uracil from SSU9 (ssDNA) (lanes 2 and 3). However, when the same sequence is presented in dsDNA context, it is utilized as substrate (lanes 5 and 6). Addition of Ugi to this reaction did not result in any inhibition of MtuUdgB activity (compare lanes 5 and 6).

When SSU9 was annealed to complementary DNA oligomers to bring uracil against G, A, C or T and used as substrates for MtuUdgB, uracil excision from these duplexes was comparable (Fig. 4B). These observations suggest that the nature of the nucleotide opposite to the target uridine in the substrate is not important. To check if the presence of a nucleotide opposite to uracil is at all important, we used U-bulge, a tetraloop hairpin DNA containing uracil in a bulge in the stem region (Table 1). Interestingly, MtuUdgB processed the U-bulge oligomer and its processing was seen to gradually increase with time (Fig. 4B).

3.6. Excision of other damaged bases by MtuUdgB

To check the specificity of MtuUdgB towards damaged base recognition, we analyzed for excision of hypoxanthine (Hx), etheno-C (εC), dihydroxy U (DHU), 5-OH-C and etheno-A (εA) containing dsDNAs by MtuUdgB. MtuUdgB excised Hx from dsDNA (Fig. 4C lanes 2, 4 and 6). The efficiency of its excision was poor from Hx:T or Hx:C pair (lanes 4 and 6) but better from an Hx:G pair (lane 2). Similarly, MtuUdgB excised εC (lane 8) but excision of 5-OH-C, DHU, and εA was undetectable (data not shown). Although a more detailed analysis with these substrates is necessary, the observations clearly show that like FaeUdgB, MtuUdgB also possesses broad substrate specificity.

3.7. Electrophoretic mobility shift assays and uracil excision by immobilized MtuUdgB

Incubation of GU9 or Gap9 with MtuUdgB resulted in a distinct slow migrating band on the native gel (Fig. 5A, compare lanes 1 and 2 with lanes 3 and 4, respectively). Such a band was absent from the lanes wherein similar analysis was performed with SSU9 (lanes 5 and 6). Elution of DNA from the bands corresponding to the free GU9 and its complex with MtuUdgB under alkaline conditions showed that while the fast migrating band predominantly contained intact GU9 (Fig. 5B, lanes 1 and 2), the slow migrating band corresponded to AP-DNA product of GU9, susceptible to treatment with alkali (lane 3). To further our understanding of the remarkable property of MtuUdgB remaining bound to the AP-DNA, we carried out reaction with MtuUdgB immobilized on Ni-NTA beads. As shown in Fig. 5C, the DNA recovered from beads migrated as product (lanes 9–14) and the one recovered from the supernatant, as the unutilized substrate (lanes 2–7). Taken together, these observations show that under the reaction conditions, the product (AP-DNA) release from MtuUdgB was not significant.

3.8. Inhibition of MtuUdgB by its products

To investigate the effect of reaction products on uracil excision by MtuUdgB, reactions were carried out using GU9. Addition of Gap9 (Fig. 6, lanes 3–5) but not of ap-bulge (lanes 6–8) or SSap9 (lanes 11–13) to the reactions (prior to addition of the enzyme) decreased uracil excision by MtuUdgB. These observations show that AP-DNA, when present in a fully double-stranded context, binds to MtuUdgB efficiently and inhibits binding of the substrate to the enzyme. Interestingly, inclusion of uracil, the other product of the MtuUdgB reaction, in the reactions had no inhibitory effect on it even at a concentration of 12 mM (lanes 16–21).
Fig. 3 – (A) Alignment of *M. tuberculosis* (*Mtu* UdgB; Rv1259) UdgB and *Thermus thermophilus* (*Tth* UdgA, TTHA1149) UdgA. Identical residues are shaded black whereas the similar residues are shaded gray. Sequence alignments were done using ClustalW, and BOXSHADE was used to obtain the shaded schematic representation (www.ch.embnet.org). Cysteines known to participate in Fe–S cluster formation in *Tth* UdgA are denoted by asterisks on top of cysteines. Class defining motifs A and B are underlined. (B) UV–vis scan of *Mtu* UdgB. The absorption peak at \( \sim 420 \) nm is indicated by an arrow. (C) Effect of treatment of *E. coli* (BW310, ung\(^{-}\)) cells harboring vector (pTrc99C, lanes 3–6), pTrc*Mtu* Ung (lanes 7–10) or pTrc*Mtu* UdgB (lanes 11–14) with indicated amounts of NaNO\(_2\) at pH 5.0 (Section 2). Reactions containing 3.2 \( \mu \)g (vector, lanes 3–6; and *Mtu* UdgB, lanes 11–14) or 160 ng (*Mtu* Ung) of cell-free extracts and 5 pmol GU9 (10,000 cpm) were incubated at 37°C for 15 min (vector, and *Mtu* UdgB) or 10 min (*Mtu* Ung), treated with NaOH, resolved on 15% polyacrylamide-8M urea gels, visualized and quantified using BioImage Analyzer. Substrate and product bands are indicated with S and P, respectively. Lanes 1 and 2 are substrate alone and pure *Mtu* UdgB control lanes. Activities of \( \beta \)-lactamase (plasmid copy number control) in unit protein contents of cell-free extracts are as indicated.

3.9. Homologous and heterologous cellular factor(s) enhance turnover by *MtuUdgB*

The observation that AP-DNA in double-stranded context remained bound to the enzyme suggested that in cellular milieu; product release from the enzyme may be facilitated by other factors. To investigate for such a possibility, we made use of cellular extracts prepared from *M. tuberculosis* and *E. coli* BW310 (ung\(^{-}\)). To inhibit Ung (family 1 UDG), the *M. tuberculosis* cell-free extract was treated with excess Ugi. As seen earlier in Fig. 1A, incubation of SSU9 with Ugi-treated *M. tuberculosis* extracts completely inhibited endogenous Ung activity (Fig. 7A, compare lanes 3 and 4). However, supplementation of *MtuUdgB* reactions with Ugi-treated *M. tuberculosis* extracts completely inhibited endogenous Ung activity (Fig. 7A, compare lanes 3 and 4). However, supplementation of *MtuUdgB* reactions with Ugi-treated *M. tuberculosis* extracts (2 \( \mu \)g), resulted in an increase in uracil excision from 32% to 57% over the incubation period of 1 h (compare lanes 5–8 with 13–16, respectively). Incubation of GU9 with the same amounts of Ugi-treated *M. tuberculosis* extracts alone did not reveal a significant activity of uracil excision (lanes 9–12) suggesting that the uracil excision activity in lanes 13–16 corresponded to the externally added *MtuUdgB*. It may be noted that the endogenous *MtuUdgB* activity is a minor activity whose detection requires high sensitivity assays such as the one shown in Fig. 1, wherein 10 \( \mu \)g of cell-free extracts were used along with the high specific activity radiolabeled substrate (unlike the standard reactions wherein radiolabeled substrate is used as a tracer). Interestingly, a time dependent increase in processing of GU9 by *MtuUdgB* was also observed in the presence of heterologous extracts prepared from *E. coli* BW310 (ung\(^{-}\)) (Fig. 7B). The observation that either homologous or heterologous cellular extracts enhanced processing of GU9 by *MtuUdgB*, suggests participation of other protein(s) in *MtuUdgB* mediated excision of uracil in the cellular milieu.

3.10. Rescue of an ung defect in *E. coli* by *MtuUdgB*

The observation that heterologous factors from *E. coli* collaborate with *MtuUdgB* allowed us to investigate biological significance of *MtuUdgB* in *E. coli*. We assayed for the mutator phenotype of an ung\(^{-}\) strain of *E. coli* MG1655 (ung::kan) harboring the vector alone or the expression constructs for Ung or *MtuUdgB* by scoring for the colonies that arise on
Fig. 4 – Substrate specificity of MtuUdgB. (A) Reactions containing 0.1 pmol (10,000 cpm) of SSU9 in single-stranded form (ss, lanes 1–3) or in double-stranded form (ds, lanes 4–6) were either not treated (lanes 1 and 4) or treated (lanes 2, 3, 5 and 6) with MtuUdgB (50 ng, ~1.5 pmol) in the absence (lanes 2 and 5) or presence of Ugi (lanes 3 and 6) at 37 °C for 1 h. (B) Effect of complementary base on uracil excision by MtuUdgB. Reactions (70 μl) containing 35 pmol (70,000 cpm) substrates as indicated, 0.35 μg MtuUdgB was added, and aliquots (10 μl) were withdrawn at the indicated times. All reactions were treated with NaOH, resolved on 15% polyacrylamide-8M urea gels, visualized and quantified using BioImage Analyzer. Product formed with respect to time of reaction were plotted for various substrates as shown. (C) Reaction of MtuUdgB with DNA oligomers containing unusual nucleosides. Oligomers (0.1 pmol, 10,000 cpm) were annealed with complementary oligomers (as shown) and either not supplemented (odd numbered lanes) or supplemented with MtuUdgB (100 ng, even numbered lanes) in 20 μl volumes at 37 °C for 1 h, treated with NaOH, resolved on 15% polyacrylamide-8M urea gels and visualized using BioImage Analyzer. Substrate and product bands are indicated with S and P, respectively.

Table 2 – Determination of mutator phenotypes by scoring for rifampicin resistance colonies

<table>
<thead>
<tr>
<th>Escherichia coli strain</th>
<th>Abundance of appearance of RifR colonies (×10^−7)</th>
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<tbody>
<tr>
<td>MG1655 (Amp^R)</td>
<td>3.2 ± 1.3</td>
</tr>
<tr>
<td>MG1655 (ung::kan) + pTrc99C</td>
<td>11.0 ± 1.5</td>
</tr>
<tr>
<td>MG1655 (ung::kan) + pTrcMtuUdgB</td>
<td>3.3 ± 0.8</td>
</tr>
<tr>
<td>MG1655 (ung::kan) + pTrcMtuUng</td>
<td>3.1 ± 1.0</td>
</tr>
</tbody>
</table>

rifampicin containing plates. As expected, in these assays, the ung^−^ strain resulted in a mutator phenotype which was ~3.5-fold higher than that of the wild type strain (Table 2). Interestingly, complementation of the ung^−^ strain with multicopy plasmid (pTrc99c based) borne copies of MtuUdgB or MtuUng rescued the mutator phenotype of E. coli MG1655 (ung::kan) to the extent observed for its wild-type counterpart. Further, while the sequence analysis of the rifampicin resistance determining region (RRDR) of the rpoB gene from the ung^−^ strain showed occurrence of C to T mutations (10 out of 10, Table 3), that of the RRDR locus from the MtuUdgB complemented strain showed 6 out of 12 mutations as C to T. This frequency (50%) of C to T mutations was the same as obtained upon complementation of the strain with MtuUng wherein 5 out of 10 mutations (50%) were C to T. Importantly, these observations show that even though MtuUdgB has broad substrate specificity (Fig. 4C), its uracil processing activity, is relevant from in vivo perspective and could serve as a backup UDG.

Table 3 – Effect of expression of MtuUdgB and MtuUng on change in mutation spectrum of rifampicin resistance determining region (RRDR) of Escherichia coli (ung^−^)

<table>
<thead>
<tr>
<th>E. coli strain</th>
<th>RRDR sequences analyzed</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C.G to T:A</td>
<td>A:T to T:A</td>
</tr>
<tr>
<td>MG1655 (ung::kan) + pTrc99C</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>MG1655 (ung::kan) + pTrcMtuUdgB</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>MG1655 (ung::kan) + pTrcMtuUng</td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>
Fig. 5 – Interaction of MtuUdgB with DNA. (A) Complexes of MtuUdgB (120 ng) with ~0.1 pmol of 5'-32P-end labeled GU9 (lane 3), Gap9 (lane 4) or SSU9 (lane 6) were formed by incubating the mixes on ice for 15 min and analyzed on 10% native PAGE. Lane 1, 2 and 5 are control lanes with free GU9, Gap9 and SSU9, respectively. (B) Analysis of DNA oligomers eluted from gel pieces under alkaline conditions, on 15% polyacrylamide-8M urea gels. Lanes 1 and 2 of the panel B show the analysis of the free oligo corresponding to lanes 1 and 3 of panel A; and the lane 3 of panel B shows the oligo corresponding to the complex in lane 3 of panel A. (C) Interaction of dsDNA with MtuUdgB immobilized on Ni-NTA beads. Aliquots of MtuUdgB bound beads were incubated with radiolabeled GU9 (5 pmol, 10,000 cpm) for varying times as indicated and separated into supernatant (lanes 2–7) and beads (lanes 9–14) (Section 2) and taken up in the sample buffer. Lanes 1 and 8 are controls where radiolabeled GU9 was mixed with the Ni-NTA beads alone and separated into supernatant (lane 1) and beads (lane 8) after incubation for 15 min. All samples were analyzed on 15% polyacrylamide-8M urea gel and visualized by BioImage Analyzer. Bands corresponding to substrate (S) and product (P) are as indicated.

Fig. 6 – Product inhibition of MtuUdgB. Aliquots of MtuUdgB (50 ng, ~1.5 pmol) were mixed with varying amount (pmol) of Gap9 (lanes 3–5), ap-bulge (lanes 6–8), SSap9 (lanes 9–13) or uracil (lanes 14–21) as indicated, supplemented with GU9 (5 pmol, 10,000 cpm for each reaction), incubated at 37°C for 15 min, treated with NaOH and analyzed on 20% polyacrylamide-8M urea gels. Bands corresponding to substrate (S) and product (P) are as shown.

4. Discussion

The integrity of DNA is constantly under threat of damages inflicted upon it by the normal physiological processes of cells and by environmental agents [4]. To maintain the genomic integrity, cells respond to damages through a complex network of DNA repair systems [32]. Among these, the base excision repair (BER) pathway is a highly evolved process of specific recognition of the damaged base and its excision by cleaving the N-glycosidic bond between the base and the sugar. In the case of mono-functional DNA glycosylases such as UDGs, the AP sites so generated are further acted upon, in a sequential manner, by AP-endonuclease, dRPase, DNA
polymerase and ligase to repair the damage in a reaction [33]. Our studies in this report were aimed to identify a novel dsDNA specific UDG activity, \( MtuUdgB \) in \( M. \) tuberculosis and to understand the salient features of substrate specificity and the mechanism of action of this family 5 UDG.

\( MtuUdgB \) shares features that overlap with those of the family 1 and family 2 UDGs. The property of excision of U from its pairs with any other base as well as from the single-nucleotide bulge (Fig. 4) is similar to the property of family 1 UDGs (Ung), which do not recognize any base in the complementary strand. On the other hand, the requirement of uracil in dsDNA context by \( MtuUdgB \) is very similar to that of the family 2 UDGs (MUG or dsUDG). However, the reasons for dsDNA requirement by the family 2 and family 5 UDGs are likely to be quite different. For excision of U or T, dsUDG directly recognizes the G opposite to U or T [8]. As the nature of the base opposite to the excised uracil is not crucial for excision by \( MtuUdgB \) (Fig. 4), direct recognition of the opposite base by the family 5 UDGs is unlikely to constitute an essential element in the mechanism of their action. Although, recognition of the sugar (to which the opposite base is attached) and the flanking phosphates has not been directly ruled out, the fact that uracil present as single-nucleotide bulge in dsDNA (lacking the 'opposite nucleotide') is excised, suggests that recognition of the nucleotide opposite uridine, if any, constitutes a relaxed requirement. Further, in contrast to the family 1 UDGs, \( MtuUdgB \) possesses broad specificity towards bases it excises from dsDNA. Among the substrates we tested, \( MtuUdgB \) (like \( PaeUdgB \)) excised hypoxanthine and ethenocytosine (in addition to uracil) from dsDNA (Fig. 4C). Thus, it may be speculated that to accommodate damaged purine or pyrimidine bases, the substrate-binding pocket in the family 5 UDGs is large. Consequently, uracil binding in this pocket could be weak. In fact, unlike the family 1 UDGs (which are extraordinarily specific for uracil in DNA and are inhibited by free uracil), \( MtuUdgB \) is not inhibited by free uracil even at 12 mM level (Fig. 6).

The observation that purified \( MtuUdgB \) displays stable binding to AP-DNA, suggests involvement of a larger network of interactions between \( MtuUdgB \) and the dsDNA in the vicinity of uracil (or AP-site). Further support to this hypothesis comes from the observation that uracil present at any
of the tetra-loop positions (occurring within a bent and the strained sugar phosphate backbone [34]) is not excised by MtuUdgB (data not shown). Whether, the interactions between the enzyme and the substrate are guided solely through contacts with the phosphate groups in the DNA backbone [35] or unspecific contacts in the major or the minor groves of the double-stranded DNA is unclear at present. However, considering that uracil excision occurs from dsDNA of different sequences, the family 5 UDGs may establish contacts with the backbone phosphates of both the strands in dsDNA at defined locations. In such a scenario, distortions in the DNA duplex would weaken its affinity with the enzyme. In fact, the ap-bulge DNA does not inhibit MtuUdgB activity (Fig. 6). Also, the U-bulge DNA is a poor substrate for MtuUdgB (Fig. 4). In addition to interactions via phosphates, sequence-independent interactions with dsDNA could also be effected via the contacts in the minor groove [36].

Why should MtuUdgB remain bound to DNA even after uracil has been excised? As has been proposed earlier for the human UNG (a family 1 UDG) [16], we believe that a major purpose for this could be to protect the AP-sites before they are recognized by the downstream repair proteins (AP-endonuclease, dRPase, DNA polymerase and ligase). The unprotected AP-sites in DNA can be highly mutagenic [37]. The observation that in the presence of cellular factors, the turnover by MtuUdgB increases (Fig. 7), supports this model of base excision repair and offers us with the first eubacterial system to study the mechanism of such a pathway initiated by MtuUdgB. This property of the repair enzyme may be physiologically vital, especially for an intracellular pathogen such as M. tuberculosis where consequences of unprotected AP-sites in DNA could be quite serious for survival and/or reactivation of the pathogen inside the hostile environment of the host macrophages.

While data shown in Fig. 3 suggest that MtuUdgB contains an iron–sulfur cluster, the exact structure of the Fe–S cluster (4Fe–4S, 3Fe–4S, 2Fe–2S, Fe–(Cys)4) is unclear. Recently, three-dimensional structure of ThhUgdA [10] showed that it contained 4Fe–4S cluster which co-ordinates four cysteines. Sequence comparison between ThhUgdA and MtuUdgB shows that the four cysteines between the two UDGs are conserved (Fig. 3A). It is known that Fe–S cluster proteins play an important role in redox regulation [30,38]. Loss of MtuUdgB activity in total extracts of E. coli cells upon treatment with acidified nitrite (known to disrupt Fe–S clusters) suggests that the presence of Fe–S cluster plays a major role in structure/function of MtuUdgB. It may also be noted that, release of Fe–S clusters from various proteins, to give rise to apo-protein and rebounding of reformed Fe–S clusters to the apo-protein under different physiological conditions, are known to allow regulation of biochemical activity of Fe–S cluster containing proteins [30]. These proteins also serve to bind free iron, which would otherwise be highly reactive and damage DNA. Such a physiological role would be relevant for mycobacteria inside macrophages. Further, it is tempting to propose that the thermo-tolerant property of MtuUdgB may well be a consequence of the presence of Fe–S cluster in it. We are presently involved in determination of the three dimensional structure of MtuUdgB to allow us detailed understanding of its structure and function.

Finally, the G+C rich nature of mycobacterial genomes and the hostile habitats of the host macrophages where the pathogenic species survive suggests that these bacteria must possess robust mechanisms to repair their genome. The genome sequence of mycobacteria has revealed [26] that these organisms lack homologs of proteins that constitute mismatch repair pathway. Thus, in mycobacteria, base excision repair (BER) and nucleotide excision repair (NER) pathways constitute the major means to mend DNA damages [39]. The crucial nature of Ung for survival of mycobacteria in mouse macrophages has already been demonstrated [16]. MtuUdgB has broad substrate specificity. However, the observation that it rescues C to T mutator phenotype of ung− E. coli suggests that in vivo MtuUdgB may play a primary role as uracil DNA glycosylase in avoiding the occurrence of C to T mutations under adverse physiological conditions. Thus, the characterization of a thermo-tolerant UDG (MtuUdgB) from M. tuberculosis is an important step in furthering our understanding of the overall mechanism of uracil excision repair pathway in this important bacterium. Such studies are vital to target important pathways to control bacterial growth as well as to design attenuated strains by genetic manipulations.

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