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The oxidative DNA glycosylases of *Mycobacterium tuberculosis* exhibit different substrate preferences from their *Escherichia coli* counterparts

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Abstract

The DNA glycosylases that remove oxidized DNA bases fall into two general families: the Fpg/Nei family and the Nth superfamily. Based on protein sequence alignments, we identified four putative Fpg/Nei family members, as well as a putative Nth protein in *Mycobacterium tuberculosis* H37Rv. All four Fpg/Nei proteins were successfully overexpressed using a bicistronic vector created in our laboratory. The MtuNth protein was also overexpressed in soluble form. The substrate specificities of the purified enzymes were characterized *in vitro* with oligodeoxynucleotide substrates containing single lesions. Some were further characterized by gas chromatography/mass spectrometry (GC/MS) analysis of products released from γ -irradiated DNA. MtuFpg1 has a substrate specificity similar to that of EcoFpg. Both EcoFpg and MtuFpg1 are more efficient at removing spiroiminodihydantoin (Sp) than 7,8-dihydro-8-oxoguanine (8-oxoG). However, MtuFpg1 shows a substantially increased opposite base discrimination compared to EcoFpg. MtuFpg2 contains only the C-terminal domain of an Fpg protein and has no detectable DNA binding activity or DNA glycosylase/lyase activity and thus appears to be a pseudogene. MtuNei1 recognizes oxidized pyrimidines on both double-stranded and single-stranded DNA and exhibits uracil DNA glycosylase activity. MtuNth recognizes a variety of oxidized bases, including urea, 5,6-dihydrouracil (DHU), 5-hydroxyuracil (5-OHU), 5-hydroxycytosine (5-OHC) and methylhydantoin (MeHyd). Both MtuNei1 and MtuNth excise thymine glycol (Tg); however, MtuNei1 strongly prefers the (5*R*) isomers, whereas MtuNth recognizes only the (5*S*) isomers. MtuNei2 did not demonstrate activity *in vitro* as a recombinant protein, but like MtuNei1 when expressed in *Escherichia coli*, it decreased the spontaneous mutation frequency of both the *fpg mutY nei* triple and *nei nth* double mutants, suggesting that MtuNei2 is functionally active *in vivo* recognizing both guanine and cytosine oxidation products. The kinetic

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parameters of the MtuFpg1, MtuNei1 and MtuNth proteins on selected substrates were also determined and compared to those of their *E. coli* homologs.

Keywords

Base excision repair; Oxidative DNA glycosylases; *Mycobacterium tuberculosis*

1. Introduction

Oxidative damage to DNA is produced by endogenous reactive oxygen species (ROS) generated during normal cellular metabolism and by exposing cells to ionizing radiation and other free radical-generating systems. These lesions include single strand breaks, abasic sites and a plethora of oxidative base damages that can be potentially mutagenic and/or cytotoxic (for a review see [1]). The DNA glycosylases function in the first step of the base excision repair (BER) pathway responsible for removing oxidatively induced base damages from DNA (for reviews see [2–5]). A DNA glycosylase cleaves the N-glycosyl bond between the sugar and the damaged base creating an abasic (AP) site. Most DNA glycosylases that recognize oxidized bases are bifunctional and contain lyase activity which then cleaves the phosphodiester backbone at the AP site leaving either an α , β -unsaturated aldehyde (β -elimination) or a phosphate (β , δ -elimination) group (for reviews see [3,6]). The DNA glycosylases that remove oxidative DNA damages fall into two general families based on structural and sequence homology, the Fpg/Nei family and the Nth superfamily (for reviews see [7,8]). The Fpg/Nei family contains formamidopyrimidine DNA glycosylase (Fpg, also called MutM), which mainly recognizes 7,8-dihydro-8-oxoguanine (8-oxoG), 4,6-diamino-5-formamidopyrimidine (FapyA) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG) and endonuclease VIII (Nei), which removes oxidized pyrimidines and FapyA (for reviews see [2–5,9]). The Nth superfamily includes endonuclease III (Nth), which has overlapping substrate specificity with Nei and is primarily responsible for removing oxidized pyrimidines; Ogg, whose primary substrates are 8-oxoG and FapyG paired with C; MutY, a monofunctional glycosylase that removes A when paired with 8-oxoG, and AlkA, also a monofunctional glycosylase that removes alkylated bases (for reviews see [2–5]). Nth is found in the three major kingdoms, bacteria, eukaryotes and archaea. While Fpg proteins are widely distributed among the bacteria and plants, Nei homologs are sparsely distributed across phyla, and are only found in γ -proteobacteria, actinobacteria and metazoans [4].

Oxidative stress also plays an important role in the host's innate immune response. Macrophages generate ROS and reactive nitrogen species (RNS) resulting in lethal damage to the DNA of pathogenic microorganisms. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, which is responsible for 3 million deaths annually and causes latent infection in one-third of the world's population, manages to survive and replicate in its host's macrophages. Therefore, the ability to repair DNA damages caused by exposure to ROS in macrophages is likely to play a particularly important role in pathogen proliferation or colonization conferring a virulence advantage for mycobacteria, as has been observed with other pathogens [10,11].

The DNA repair mechanisms of *M. tuberculosis*, as well as its response to DNA damage has distinguishing features compared to some other bacteria. First, the genome sequences of *M. tuberculosis*, *Mycobacterium leprae*, and *Mycobacterium smegmatis* indicate that these microbes lack the mismatch repair pathway [12,13]. Furthermore, *M. tuberculosis* regulates most of its inducible DNA repair genes using a novel RecA-independent mechanism, even though in most bacterial systems studied so far a RecA-dependent mechanism is the major DNA damage response mechanism [14,15]. *M. tuberculosis* possesses genes for nucleotide excision repair (NER), BER, recombination and the SOS response [12]. Among the major

DNA repair pathways, the BER and the NER pathways may contribute significantly to maintaining the genomic integrity of mycobacteria. The role of the NER pathway in *M. tuberculosis* has been examined by using *uvrB* mutants, which were highly susceptible to acidified nitrite and UV light but not to several sources of reactive oxygen intermediates (ROI) [16]. They were also markedly attenuated for survival in mice. This attenuation was reversed in mice lacking either nitric oxide synthase 2 (iNOS) or both iNOS and phagocyte oxidase, further indicating the important role that the NER pathway plays in resisting both NOS and ROS. *Ung* deficiency led to an enhanced sensitivity to reactive nitrogen intermediates (RNI) and a decrease in survival of *M. smegmatis* and *Pseudomonas aeruginosa* in macrophages, which underscores the importance of the BER pathway for these two high G+C gram-positive organisms [17]. Disrupting the *fpg* gene in *M. smegmatis* enhanced the spontaneous mutation frequency and made the microbe more susceptible to hydrogen peroxide [18]. Using transposon site hybridization (TraSH), members of the BER pathway: endonuclease IV (*nfo*), uracil glycosylase (*ung*) and exonuclease III (*xthA*) were shown to be important for the *in vivo* growth of *M. tuberculosis* during initial infection [19]; however, no DNA glycosylases that remove oxidatively modified DNA bases were shown to be necessary for its survival *in vivo* probably due to the redundant activities of these enzymes [20].

Four putative Fpg/Nei family members, as well as one putative Nth protein were identified in *M. tuberculosis* H37Rv, MtuFpg1, MtuFpg2, MtuNei1, MtuNei2 and MtuNth [21]. Some progress has already been made towards defining the biochemical properties of these DNA glycosylases. Sidorenko *et al.* cloned two of the four Fpg/Nei enzymes: MtuFpg2 and MtuNei1 (Mtu-Nei2 in their study) and biochemically characterized the partially purified MtuNei1 [22]. More recently, Olsen *et al.* partially characterized the activity of purified MtuFpg1 and pointed out that the *in vivo* transcription of the gene encoding MtuFpg1 decreased with decreasing the length of the intergenic repeat upstream to the gene [23]. However, no extensive biochemical characterization of the mycobacterial DNA glycosylases was done.

We have successfully overexpressed and purified all four MtuFpg/Nei proteins and the MtuNth protein. The substrate specificities of MtuFpg1, MtuNei1 and MtuNth were characterized and compared to their *E. coli* counterparts *in vitro* with oligodeoxynucleotide substrates containing single lesions. They were further characterized by gas chromatography/mass spectrometry (GC/MS) analysis of products released from γ -irradiated DNA. The kinetic parameters of the MtuFpg1, MtuNei1 and MtuNth proteins on selected substrates as well as those of their *E. coli* homologs were also determined. Both Nei paralogs, MtuNei1 and MtuNei2, were also shown to be functional *in vivo*. Taken together, our data indicate that the DNA glycosylases of *M. tuberculosis* that recognize oxidatively induced DNA damages have substrate specificities that overlap but are distinct from their *E. coli* counterparts. The exception is MtuFpg2 that appears to be a pseudogene.

2. Materials and methods

2.1. Materials

The genomic DNA of *M. tuberculosis* H37Rv was kindly provided by Dr. Karin Eiglmeier and Dr. Nadine Homoré (Institute Pasteur, Paris, France). The gene sequences were retrieved from GenBank for *M. tuberculosis* H37Rv (Rv0944: gi|15608084, Rv2464c: gi|15609601, Rv2924c: gi|15610061, Rv3297: gi|1877352, Rv3674c: gi|57117142). Based on the sequence alignment, two of the five were identified as putative Fpg homologs: MtuFpg1 (Rv2924c) and MtuFpg2 (Rv0944), two fell into the Nei clade: MtuNei1 (Rv2464c) and MtuNei2 (Rv3297), and one is an Nth homolog: MtuNth (Rv3674c).

2.2. Protein expression and purification

The genes encoding MtuFpg1 (*Rv2924c*) and MtuFpg2 (*Rv0944*) were individually cloned between the EcoRV and XhoI sites of the bicistronic vector pET30a-ORF6, as previously described to allow active expression of the C-terminal hexa-his tagged proteins [24]. In order to avoid the potential inhibitory effect of rare codons during translation, the pRARE2 plasmid (Novagen, Madison, WI) carrying seven rare-codon tRNA genes (GGA, CUA, AUA, CCC, AGG, AGA, CGG) was co-transformed with each expression plasmid into *E. coli* ER2566 (Fpg⁻). The expression of MtuFpg1 and MtuFpg2 were respectively induced with 0.5 mM IPTG in Luria Broth (LB) medium (50 µg/ml kanamycin, 34 µg/ml chloramphenicol and 10 µM ZnSO₄) at an OD₆₀₀ of 0.5. The cultures were further grown at 20 °C for overnight. To purify these target proteins, cells from 2 liters of induced cultures were harvested by centrifugation and re-suspended in chelating column Buffer A (50 mM sodium phosphate buffer (pH 8.0), 100 mM NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol) supplemented with 1 mM PMSF, 10 mM benzamidine and 1% deoxyribonuclease I (Invitrogen). The clear cell lysates were harvested after sonication and centrifugation then loaded onto a 5 mL HiTrap chelating HP column (GE Healthcare, Piscataway, NJ) using ÄKTA purifier (GE Healthcare). The target protein was eluted with a linear gradient of 0–100% chelating column Buffer B (50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 500 mM imidazole (pH 8.0), 10% (v/v) glycerol, 5 mM β-mercaptoethanol) in 20 column volumes. Fractions containing the protein were identified by SDS-PAGE, pooled and loaded onto a 1 mL HiTrap SP FF column (GE Healthcare) with SP column buffer A (20 mM HEPES (pH 7.6), 50 mM NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol). The protein was further eluted with a linear gradient of 0–100% SP column Buffer B (20 mM HEPES (pH 7.6), 1 M NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol). The purified target protein was pooled and stored in the MtuFpg storage buffer (20 mM HEPES KOH (pH 7.6), 250 mM NaCl, 2 mM TCEP, 50% glycerol) at –20 °C.

The genes for MtuNei1 (*Rv2464c*) and MtuNei2 (*Rv3297*) were optimized with *E. coli* preferred codons and chemically synthesized by GeneScript Corporation (Piscataway, NJ). The two synthesized genes were also cloned between the EcoRV and XhoI sites of pET30a-ORF6 vector. Expression was induced in *E. coli* Arctic Express (DE3) cells (Stratagene, Cedar Creek, TX) with 1 mM IPTG in the presence of 10 µM ZnSO₄. The target protein was first purified with a 5 mL HiTrap chelating HP column (Buffer A: 50 mM sodium phosphate buffer (pH 8.0), 500 mM NaCl, 10% (v/v) glycerol, 15 mM β-mercaptoethanol; Buffer B: 50 mM sodium phosphate buffer (pH 8.0), 500 mM NaCl, 500 mM imidazole (pH 8.0), 10% (v/v) glycerol, 15 mM β-mercaptoethanol) followed by a 1 mL HiTrap phenyl HP column (GE Healthcare) (Buffer A: 50 mM sodium phosphate buffer (pH 8.0), 1 M (NH₄)₂SO₄, 10% (v/v) glycerol, 15 mM β-mercaptoethanol; Buffer B: 50 mM sodium phosphate buffer (pH 8.0), 10% (v/v) glycerol, 15 mM β-mercaptoethanol). The purified protein was then stored in the MtuNei storage buffer (20 mM HEPES NaOH (pH 7.6), 150 mM NaCl, 2 mM TCEP, 50% glycerol) at –20 °C.

The MtuNth (*Rv3674c*) gene was cloned between the NdeI and SalI sites of the pET28a vector in order to express the protein fused with an N-terminal hexa-his tag. To avoid the potential inhibitory effect of rare codons during translation, the pRARE2 plasmid (Novagen) was co-transformed with the pET28a containing MtuNth. The expression of MtuNth was induced in *E. coli* ER2566 (Fpg⁻) with 0.1 mM IPTG at 20 °C overnight. Similar to MtuNei1 and MtuNei2, the MtuNth protein was also purified with a 5 mL HiTrap chelating HP column (GE Healthcare) followed by a 1 mL HiTrap SP FF column. The purified MtuNth was stored in Nth storage buffer (50 mM HEPES-NaOH (pH 7.6), 50 mM NaCl, 2 mM TCEP, 50% glycerol).

The identity of each purified protein was confirmed by N-terminal protein sequencing (Biomolecular Resource Facility, The University of Texas Medical Branch). The control

enzymes (EcoFpg, EcoNei, NEIL1 and EcoNth) were prepared in our laboratory as previously described [25]. The protein concentrations were determined with Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL).

2.3. Substrates

The 35-mer and 51-mer oligodeoxynucleotides carrying various oxidatively induced lesions and the complementary strands were purchased from Midland Certified Reagent Co. (Midland, TX). The sequence of the 35-mer damage-containing oligodeoxynucleotides is: 5' - TGTCAATAGCAAGXGGAGAAGTCAATCGTGAGTCT - 3', where X = 7,8-dihydro-8-oxoguanine (8-oxoG), thymine glycol (Tg), 5,6-dihydrouracil (DHU), 5,6-dihydrothymine (DHT), 5-hydroxyuracil (5-OHU), 5-hydroxycytosine (5-OHC), uracil (U) and furan (F). Another 35-mer complementary strand was designed based on the work of Dou *et al.* [26]: 5' - AGACTCACGATTGACTTCTCCGAACGATAACTGT - 3'. This oligodeoxynucleotide contained a noncomplementary sequence for annealing to the 5-OHU-containing 35-mer to generate the 3' forked structure. A 30-mer oligodeoxynucleotide carrying either of the two *cis* isomers of Tg (5' -CTCGTCAGCATCTZCATCATACAGTCAGTG - 3', where Z = 5R -Tg ((5R,6S) plus (5R,6R)) or 5S-Tg ((5S,6R) plus (5S,6S)) as well as the complementary strand was synthesized [27,28]. Three 51-mer oligodeoxynucleotides carrying 8-oxoG, U or 5-OHU at position 26 from the 5'-end and a series of complementary strands were synthesized as previously described [29]. These complementary strands contained the sequences for either creating base-paired duplex substrates or producing bubble structures with 5, 11 or 19 unpaired bases flanking the damage. A 30-mer oligodeoxynucleotide was synthesized carrying either guanidinohydantoin (Gh) or spiroiminodihydantoin (Sp) (5' - TGTTCATCATGCGTCYTCGGTATATCCCAT - 3', where Y = Gh or Sp) [30].

1 pmol of each damage-containing strand was 5' end-labeled with [γ -³²P] ATP by T4 polynucleotide kinase (New England Biolabs, Beverly, MA) at 37 °C for 20 min. The reaction was terminated by addition of EDTA followed by heat inactivation. The labeled oligodeoxynucleotide was then ethanol precipitated and diluted with 9 pmol of the unlabeled lesion-containing oligodeoxynucleotide in the substrate buffer (20 mM Tris HCl (pH 8.0), 50 mM NaCl and 1 mM EDTA) and used as the single-stranded substrate. To create the duplex substrate, the lesion-containing strand was annealed to an equal amount of its complementary strand by heating to 95 °C then slowly cooling down to room temperature. To create a urea substrate, double-stranded oligodeoxynucleotides containing Tg were subjected to alkali hydrolysis by dialyzing against 40 mM sodium phosphate buffer (pH 12.0) and 1 mM EDTA for overnight then dialyzed into the substrate buffer for a further 24 hours [31]. To create an abasic (AP) site, duplex or single-stranded oligodeoxynucleotides containing U were treated with EcoUdg (New England Biolabs) at room temperature for 15 min.

2.4. Determining the active fraction of the enzyme preparations

The Schiff base assay was used to determine the active fraction of each mycobacterial enzyme preparation. An increasing amount of each enzyme preparation was incubated with 1 μ M of its optimal substrate in the presence of 50 mM of sodium borohydride (NaBH₄). The concentration of NaCl used in the Schiff base reactions was adjusted correspondingly to keep the final sodium concentration the same as in the optimal reaction buffer for each enzyme. After 30 min of incubation at 37 °C, the reactions were terminated with SDS loading buffer (50 mM Tris buffer (pH 8.0), 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 100 mM β -mercaptoethanol) and boiled for 5 min before loading on 12% SDS-PAGE. The gel bands were detected by using a phosphoimage screen (Bio-Rad, Hercules, CA) and quantified with Quantity-one (Bio-Rad). We plotted the quantity of trapped substrate formed in the Schiff base assay versus the total enzyme concentration used in the assay. The slope of the linear region before the plateau was reached indicates the active fraction of the enzyme preparation

[32]. Similarly, the active fraction of each control enzyme was determined using the molecular accessibility assay [32]. A table of the enzyme preparations used is shown in the Supplementary Material (Table S1). Notably, MtuFpg2 (*Rv0944*) showed no detectable DNA glycosylase/lyase activity hence could not form a DNA-enzyme complex in the presence of NaBH₄ (data not shown). Except for MtuFpg2 (*Rv0944*), the amount of each enzyme used in the following assays was corrected for the percent active fraction determined for each preparation.

2.5. DNA glycosylase/lyase assays

For duplex substrates, 15 nM labeled substrate was incubated with either 15 nM control enzymes or increasing amounts of the mycobacterial enzymes (1.5 nM, 15 nM or 150 nM) in their optimal reaction buffers supplemented with 0.1 mg/mL of BSA. The reaction buffers are as follows: MtuFpg1, EcoFpg, EcoNei and NEIL1 (20 mM Tris HCl (pH 8.0), 75 mM NaCl and 1 mM EDTA); MtuNei1, MtuNth and EcoNth (20 mM Tris·HCl (pH 8.0), 50 mM NaCl and 1 mM EDTA). The reactions for EcoFpg, MtuFpg1, EcoNth and MtuNth were carried out at 37 °C for 30 min. Since MtuNei1 is temperature-sensitive and unstable at 37 °C, the reactions for EcoNei, NEIL1 and MtuNei1 were carried out at room temperature for 30 min. For single-stranded substrates, 15 nM of each labeled substrate was incubated with 15 nM of each enzyme at the corresponding optimal reaction condition as described above. All the reactions were terminated with an equal volume of formamide stop loading buffer (98% formamide, 5 mM EDTA, 0.1% xylene cyanol and 0.1% bromophenol blue). The cleaved and uncleaved substrates were separated on a 12% denaturing polyacrylamide gel and quantified as above.

2.6. Electrophoretic mobility shift assays

The ³²P-labeled 35-mer duplex substrates (25 nM) containing either 8-oxoG, furan or the normal base G at the corresponding position were incubated with either 25 nM of MtuFpg1 (*Rv2924c*) or 5 μM of MtuFpg2 (*Rv0944*) in the MtuFpg1 optimal reaction buffer supplemented with 5% glycerol and 0.1 mg/mL BSA at 25 °C for 30 min. The reactions were electrophoresed on a 4% native polyacrylamide gel (4% acrylamide, 0.5×TBE, 1% glycerol) at 4 °C.

2.7. Enzyme kinetics

Kinetic parameters were determined for MtuFpg1, MtuNei1, MtuNth and their *E. coli* homologs, EcoFpg, EcoNei and EcoNth, on their representative duplex substrates. The reactions were terminated at appropriate time points with formamide stop loading buffer. Kin-Tek Rapid Quench was used when the reactions were too fast to be terminated manually. The k_{obs} and $(k_{\text{cat}}/K_{\text{m}})^{\text{app}}$ were solved under single-turnover conditions and multiple-turnover conditions respectively. Under single-turnover conditions, enzymes with concentrations higher than the dissociation constants (K_{d}) were incubated with either 0.5 nM or 5 nM labeled substrate in their optimal reaction buffers (see Section 2.5) supplemented with 0.1 mg/mL of BSA. Reactions were terminated at the appropriate time points ranging from 0.1 seconds to 30 minutes with formamide stop loading buffer. The data were analyzed with either the one phase or two phase exponential association model of Prism 4 (GraphPad Software, Inc. La Jolla, CA). Similarly, under multiple turnover conditions, either 5 nM or 10 nM enzyme was incubated with increasing amounts of substrate ([enzyme]:[substrate] ratios were 1:2, 1:5, 1:10 and 1:20 respectively in each group of data). Reactions were terminated at the appropriate time points ranging from 5 seconds to 30 minutes with formamide stop loading buffer, as described above. Initial velocities (v_0) were determined and plotted versus different substrate concentrations ([S]) with Prism 4 (GraphPad Software, Inc. La Jolla, CA). When the substrate concentration was very low, the $(k_{\text{cat}}/K_{\text{m}})^{\text{app}}$ values could be calculated using velocity (v), substrate concentration [S] and enzyme concentration [E_T]:

$$v = \left(\frac{k_{\text{cat}}}{K_m} \right)^{\text{app}} [S][E_T] \quad (\text{Eq. 1})$$

2.8. Gas chromatography/mass spectrometry (GC/MS) analysis

Calf thymus DNA was γ -irradiated with 40 Gray in N_2O saturated phosphate buffer solution to create the DNA damages as previously described [33,34]. Stable isotope-labeled analogs of the damaged DNA bases as internal standards were added to 50 μg of γ -irradiated DNA. The samples were then incubated with 2 μg of active protein (MtuFpg1, EcoFpg, MtuNth or EcoNth) in 50 μl buffer (50 mM phosphate buffer (pH7.4), 100 mM KCl, 1 mM EDTA and 0.1 mM DTT) at 37 °C for 1 h. The reactions for EcoNei and MtuNei1 were performed at room temperature. For each enzyme, the concentration used was in the linear portion of the progress curve. The subsequent GC/MS analysis was explained previously [33,35] and briefly described below. The GC/MS data were collected from three independent experiments using three independently prepared DNA substrates.

GC/MS data consist of quantities, Q_{epr} , each associated with an enzyme, e , a product, p , and a replicate index, r . The quantity of each product released by each enzyme, S_{ep} , was calculated using

$$S_{ep} = \frac{1}{|R_{ep}|} \sum_{r \in R_{ep}} Q_{epr} - \frac{1}{|R_{0p}|} \sum_{r \in R_{0p}} Q_{0pr} \quad (\text{Eq. 2})$$

where no enzyme is indicted by $e=0$, R_{ep} is the set of replicate indices, and $|R_{ep}|$ is the number of replicates [34].

2.9. Spontaneous mutation frequency assays

E. coli KL16 wild type strain was generously provided by Dr. Bernard Weiss, Department of Pathology, Emory University. KL16 $\Delta fpg::Amp \Delta nei::Cam mutY::Tet$ and KL16 $\Delta nei::Cam nth::Kan$ were created in our laboratory as described earlier [36]. The mutant strains carrying the λDE3 lysogen were generated using the λDE3 lysogenization kit (Novagen) to allow heterologous expression with pET vectors. The pET21a-ORF6 vector containing either MtuNei1 (*Rv2464c*) or MtuNei2 (*Rv3297*) and the pET28a containing the EcoNei gene were respectively electroporated into KL16 $\Delta fpg::Amp \Delta nei::Cam mutY::Tet$ (DE3) and KL16 $\Delta nei::Cam nth::Kan$ (DE3). A low-level of heterologous expression was maintained with 0.1 mM IPTG and confirmed by SDS-PAGE. The activities of EcoNei and MtuNei1 were confirmed by cell extract assays as described earlier [36,37]. Overnight cultures of each strain containing 0.1 mM IPTG and appropriate antibiotics were diluted 50-fold into fresh LB medium containing only 0.1 mM IPTG and further grown at 37 °C until an OD_{600} of 0.5. The mid-log-phase cultures were plated onto LB-agar plates with and without 100 $\mu\text{g}/\text{ml}$ of rifampin (Rif) and 0.1 mM IPTG. The Rif-resistant (Rif^R) mutant fraction for each strain was calculated.

3. Results

3.1. MtuFpg1 prefers double-stranded DNA containing oxidized purines

To determine the substrate specificity of MtuFpg1, we tested its activity on double-stranded oligodeoxynucleotides containing a single base lesion or an AP site with EcoFpg as a control. Like other Fpg proteins, MtuFpg1 is bifunctional with glycosylase and lyase activities. As shown in Fig. 1 and supplementary Fig. S1, MtuFpg1 recognizes an AP site opposite G, T and C equally well but is slightly less active on AP:A. MtuFpg1 also cleaved the phosphodiester backbone at the AP site via β , δ -elimination leaving a phosphate at the 3' end. Besides an AP site, 8-oxoG:C is the best substrate for MtuFpg1 as expected. MtuFpg1 also discriminates among the bases opposite with 8-oxoG: C > G ~ T >> A (Fig. 1 and supplementary Fig. S2), consistent with a previous report on *M. smegmatis* Fpg [18] and similar to EcoFpg [38]. Notably, while EcoFpg exhibited slower enzymatic activity on 8-oxoG:A than 8-oxoG:C [39], MtuFpg1 showed barely detectable activity on 8-oxoG:A (Fig. 1 and [23]). This observation was confirmed as shown in Fig. 2. Complete cleavage of 8-oxoG:A was achieved by increasing the amount of EcoFpg, but cleavage of 8-oxoG:A by MtuFpg1 was barely detectable even at an enzyme to substrate ratio of 10:1.

8-OxoG can be further oxidized to yield the hydantoin lesions, spiroiminodihydantoin (Sp) and guanidinohydantoin (Gh) [40,41]. Unlike EcoFpg which recognizes Gh and Sp equally well [39,42,43], MtuFpg1 prefers Sp:C over Gh:C (Fig. 1). On the other hand, similar to EcoFpg, MtuFpg1 was less active on pyrimidine substrates including DHU, 5-OHU, 5-OHC and urea. MtuFpg1 did not recognize Tg:A or DHT:A. Taken together, our biochemical data indicate that the substrate preference of MtuFpg1 is similar to that of EcoFpg, which primarily recognizes modified purines in double-stranded oligodeoxynucleotides; however, MtuFpg1 exhibits a much stronger opposite base discrimination against 8-oxoG:A than does EcoFpg.

3.2. Rv0944 encoding MtuFpg2 appears to be a pseudogene

MtuFpg2, a 16 kDa protein, contains only the intact C-terminal domain of an Fpg protein according to the sequence alignment. This protein lacks the N-terminal proline (Pro2), which is the catalytic residue for nearly all the Fpg/Nei proteins [44], the Glu3 which is highly conserved and required for glycosylase activity of EcoFpg [45], as well as the Lys53 (EcoNei position) which together with the H2TH and zinc finger motifs create strong hydrogen bonding to stabilize the damaged DNA [46,47]. The loss of these critical amino acids as well as part of the N-terminal domain in MtuFpg2 suggested that this protein had lost its original DNA glycosylase/lyase activity and, as expected, we were not able to demonstrate either activity (data not shown). Considering that MtuFpg2 contains the C-terminal domain and that this domain in Fpg/Nei proteins functions in binding to DNA [46,48–52], we tested whether MtuFpg2 contains DNA binding ability using the electrophoretic mobility shift assay (shown in supplementary Fig. S3). While MtuFpg1 bound to the duplex substrates containing 8-oxoG, furan (F) and to undamaged DNA, MtuFpg2 did not show any DNA binding ability. Since MtuFpg2 has no detectable DNA binding activity or DNA glycosylase/lyase activity, the gene encoding MtuFpg2 appears to be a pseudogene.

3.3. MtuNei1 recognizes oxidized pyrimidines in both double-stranded and single-stranded DNA

We first tested the substrate specificity of MtuNei1 on double-stranded substrates and compared its activity to EcoNei and human NEIL1 (Fig. 3A). As expected, the best substrates for MtuNei1 were Tg, DHU, urea, and an AP site. MtuNei1 cleaved the phosphodiester backbone via β , δ -elimination, as do other Fpg/Nei family members. Similar to EcoNei [53, 54] and NEIL1 [55–57], MtuNei1 exhibited weak activity on DHT, 5-OHU and 5-OHC. We did not detect any activity of MtuNei1 against 8-oxoG at the enzyme concentrations used;

however, NEIL1 did show weak activity against 8-oxoG, which is consistent with previous observations [55,56,58]. Like EcoNei [43,59] and NEIL1 [60,61], MtuNei1 could also excise Gh and Sp, which suggests a backup role for MtuNei1 in repairing oxidized purines.

Interestingly, MtuNei1 contains uracil DNA glycosylase activity (Fig. 3B); weak uracil DNA glycosylase activity was also observed for EcoNei and NEIL1 when the reactions were incubated at 37 °C instead of room temperature (data not shown). MtuNei1 was able to excise uracil (U) from both double-stranded and single-stranded substrates (Figs. 3B and 4) with higher efficiency than EcoNei and NEIL1 at both temperatures. It recognizes uracil opposite T, G and C equally well and slightly better than U:A. Uracil arises in DNA by deamination of cytosine or by incorporation of dUMP, so among these substrates, U:A and U:G are the biological substrates. This redundant uracil DNA glycosylase activity may be particularly important for maintaining the integrity of the G/C-rich genome of *M. tuberculosis*.

MtuNei1 was also capable of incising the oxidized pyrimidines, Tg, DHU, DHT, 5-OHU and 5-OHC from single-stranded oligodeoxynucleotides (Fig. 4A), but MtuNei1 was less active on single-stranded substrates than on double-stranded substrates. We quantified the activity of MtuNei1 on single-stranded oligodeoxynucleotides containing single lesions in order to compare it to EcoNei and NEIL1 (Fig. 4B). In general, NEIL1 protein excised the single-stranded substrates significantly better than either EcoNei or MtuNei1; however, MtuNei1 was significantly more active on Tg in single-stranded DNA compared to the other lesion-containing single-stranded substrates.

Since the activity of MtuNei1 on single-stranded substrates suggests a possible function in replication-associated DNA repair, we tested the activity of MtuNei1 on bubble and 3' forked structures, which mimic the transient structures generated during DNA replication and transcription, and compared it to EcoNei and NEIL1. As stated above, the three enzymes are more active on double-stranded DNA than single-stranded DNA (Fig. 5). Moreover, duplex DNA containing 5-OHU is not as good a substrate for MtuNei1 as for EcoNei or NEIL1. Each enzyme had comparable activity on the three bubble structures (Fig. 5A), although their preferences toward different-sized bubbles are different (Fig. 5A and [29]). MtuNei1 showed comparable activity on duplex DNA and the 3' forked structure, as did NEIL1 [26]; on the other hand, EcoNei preferred duplex DNA (Fig. 5B). The different cleavage levels observed with the 35-mer duplex substrates (Fig. 5A) compared to the 51-mer duplex substrates (Fig. 5B) may due to the different lengths and sequence contexts. Regardless, MtuNei1 was significantly more active on lesions in duplex DNA, bubble and 3' forked structures than on lesions in single-stranded DNA. Notably MtuFpg1 and MtuNth showed no activity against any lesion in single-stranded DNA, unlike EcoFpg which recognizes 8-oxoG in single-stranded DNA (Fig. 4A and [62,63]); essentially complete cleavage was observed for all enzymes tested on the single-stranded oligodeoxynucleotide containing an AP site (Fig. 4A).

3.4. Comparison of the kinetic parameters of the *M. tuberculosis* and *E. coli* oxidative DNA glycosylases

The kinetic parameters, k_{obs} (min^{-1}) and $(k_{\text{cat}}/K_m)^{\text{app}}$ ($\text{min}^{-1}\text{nM}^{-1}$), of MtuFpg1, MtuNei1 and MtuNth as well as their *E. coli* homologs were determined for selected double-stranded substrates under their optimal reaction conditions and shown in Table 1. Overall, the rate constants of the *M. tuberculosis* enzymes are lower than those of the *E. coli* enzymes.

MtuFpg1 showed specificity similar to EcoFpg but with the k_{obs} values about 10-fold lower than those of EcoFpg. 8-OxoG and its oxidized product Sp are much better substrates for the two Fpg enzymes than Tg and DHU. Consistent with previous observations [42], we found that EcoFpg was at least 100-fold more efficient at removing Sp than 8-oxoG probably because of the tighter binding to Sp. Under these same conditions, MtuFpg1 was only 10-fold more

efficient at removing Sp than 8-oxoG. Moreover, MtuFpg1 repaired Tg and DHU much less efficiently than EcoFpg. Therefore, MtuFpg1 appears to be more purine-specific than EcoFpg.

The kinetic rates of MtuNei1 are 100-fold lower than those of EcoNei. In terms of enzymatic specificity, 5-OHC is the worst substrate for MtuNei1 among those tested, similar to EcoNei. Although EcoNei did not show measurable activity against 8-oxoG under these conditions, the activity of EcoNei against Sp was similar to that of EcoFpg [43,64].

The kinetic parameters of MtuNth are comparable to those of EcoNth. The k_{obs} of MtuNth for 5-OHC is even higher than that of EcoNth, although the overall efficiency of MtuNth repairing 5-OHC is much lower, probably because of low binding affinity. The best substrate for MtuNth is DHU. The analysis of the single turnover kinetics of the Nei and Nth enzymes on Tg fits a double exponential model much better than a single exponential model, which suggested that these enzymes were stereospecific (see below).

3.5. GC/MS analysis of products released by the *M. tuberculosis* proteins compared to their *E. coli* counterparts

The product spectrum of damages released from γ -irradiated DNA after incubation with the mycobacterial DNA glycosylases as well as their *E. coli* homologs were determined by GC/MS (Table 2). The GC/MS data showed some new substrates for the mycobacterial DNA glycosylases and confirmed our observations with the *in vitro* biochemical and kinetics assays. Not only 8-oxoG but also FapyG and FapyA are good substrates for MtuFpg1; however, MtuFpg1 does not recognize 8-oxoA as well as EcoFpg. 8-OxoA is a good substrate for MtuNth. The activity of MtuNei1 on MeHyd, 5-OHU or FapyG is comparable to that of EcoNei. While EcoNei prefers FapyA and 5-OHC, MtuNei1 is not efficient at excising these two lesions. 5-OHC, 5-OHU and FapyA turned out to be good substrates for both MtuNth and EcoNth. The low efficiency of MtuNei1 and MtuNth in repairing Tg as suggested by GC/MS analysis is probably due to the heterogeneity of Tg produced by γ -irradiation [65].

3.6. MtuNth prefers the (5*S*) isomers of Tg, whereas MtuNei1 prefers the (5*R*) isomers

Nth proteins are oxidized pyrimidine-specific DNA glycosylases. Similar to EcoNth, the good substrates for MtuNth were DHU, urea, AP sites, 5-OHU and 5-OHC (Fig. 3A). DHT was a poor substrate for both MtuNth and EcoNth. Surprisingly, unlike EcoNth, MtuNth did not excise Tg with high efficiency (Fig. 3A). Considering that in aqueous solution Tg is a mixture of two *cis-trans* pairs of isomers ((5*R*,6*S*);(5*R*,6*R*) *cis-trans* pair and (5*S*,6*R*);(5*S*,6*S*) *cis-trans* pair) and the Tg presented in our 35-mer oligodeoxynucleotide consists predominantly of the (5*R*) *cis-trans* pair, this result may be due to the stereospecificity of MtuNth toward the (5*S*) *cis-trans* pair. Moreover, the single turnover kinetics with Tg suggested that both MtuNth and EcoNth were stereospecific (Table 1). The smaller k_{obs} numbers are associated with the predominant isomer, which suggests that both Nth enzymes prefer the (5*S*) isomers. Both Nei proteins were also stereospecific. In contrast to the Nth proteins, the large k_{obs} values obtained with MtuNei1 and EcoNei correspond to the predominant (5*R*) isomers. This is consistent with what we observed in Fig. 3 as well as with previous observations of EcoNei and EcoNth [66, 67]. We then further tested the stereospecificities of the four enzymes with the 30-mer oligodeoxynucleotide containing either the (5*R*) or the (5*S*) *cis-trans* pairs. As shown in Fig. 6, MtuNth strongly preferred the (5*S*) isomers and did not efficiently cleave the (5*R*) isomers, whereas MtuNei1 excised the (5*R*) isomers much better than the (5*S*) isomers. The enzymatic activities of MtuNth and MtuNei1 in excising the two *cis-trans* pairs of Tg are perfectly complementary, and even though MtuNth and MtuNei1 exhibit the stereospecific tendencies of their *E. coli* homologs [66,67], they are much more stereospecific (Fig. 6).

In order to further explore which amino acids of MtuNth are responsible for its stringent stereospecificity, Thr47 and Ser187 in the potential lesion recognition pocket of MtuNth were changed to Val and His respectively. However, none of the variant enzymes showed enhanced activity on (5*R*)-Tg (data not shown).

3.7. MtuNei2 can decrease the spontaneous mutation frequency of *E. coli* mutants

The *M. tuberculosis* Rv3297 gene encodes a 28.5 kDa endonuclease VIII (MtuNei2). We successfully overexpressed and purified MtuNei2 using the bicistronic vector. Unfortunately, the active portion of each MtuNei2 preparation was low, 0.5% – 1% based on our Schiff base assays (data not shown). The cell extract assays with the cell lysates expressing MtuNei2 did not give us promising results either (data not shown). We then decided to determine if MtuNei2 was able to complement the spontaneous mutation frequencies of *E. coli* mutants. Fig. 7 and supplementary Table S2 show the spontaneous mutation frequencies to Rifampin resistance (Rif^R) of wild type *E. coli*, *E. coli fpg mutY nei* triple mutants, *E. coli nei nth* double mutants, as well as these same mutants expressing either EcoNei, MtuNei1 or MtuNei2. IPTG, 0.1mM, was used to maintain a low-level of heterologous expression. The activity of MtuNei1 and EcoNei was confirmed using cell extracts with double-stranded oligodeoxynucleotides containing Tg (data not shown). Consistent with what we have previously observed [36], the *fpg mutY nei* triple mutants exhibit an extremely high spontaneous mutation frequency, about 500-fold higher than the wild type (Fig. 7A). This mutation frequency can be significantly decreased by expressing either EcoNei, MtuNei1 or MtuNei2 ($p < 0.05$) in the mutant cells. Since the triple mutant accumulates G to T transversions due to its failure to repair guanine damage, which is the biological function of Fpg and MutY [36], our data suggest that both MtuNei1 and MtuNei2 can prevent G to T transversions as does EcoNei probably by removing oxidized guanine products, such as Sp and urea. Furthermore, *nei nth* double mutants also exhibit an elevated spontaneous mutation frequency although not as high as the *fpg mutY nei* triple mutants (Fig. 7, supplementary Table S2 and [36,37]). The spontaneous mutations observed in the double mutant are solely from C to T transitions due to the inability to repair oxidized cytosines [36], which can be efficiently prevented by EcoNei, MtuNei1 and MtuNei2 (Fig. 7B). Because Nei proteins play a major role in repairing pyrimidine damage and a backup role in repairing guanine damage, their effects on decreasing mutation frequencies are greater with the *nei nth* double mutants (Fig. 7B) than the *fpg mutY nei* triple mutants (Fig. 7A). Taken together, our data suggest that MtuNei2 can function as an active DNA glycosylase removing oxidized G and C products *in vivo* therefore preventing mutagenesis.

4. Discussion

4.1. In contrast to EcoFpg, MtuFpg1 does not remove 8-oxoG when paired with A

The most notable difference between EcoFpg and MtuFpg1 is their opposite base specificities. Both enzymes recognize 8-oxoG paired with C, T and G, however, MtuFpg1 exhibits strong opposite base specificity having almost no activity on 8-oxoG:A (Figs. 1 and 2 and [23]), whereas EcoFpg retains its ability to repair 8-oxoG:A albeit with significantly lower efficiency than 8-oxoG:C [68–70]. The basis for this difference is still unknown, as MtuFpg1 exhibits significant sequence similarity to EcoFpg and contains the conserved Fpg family insertion loop (Met74, Arg109 and Phe111), of which Arg109 has been shown to contribute to the opposite base specificity [52]. Interestingly, the replicative DNA polymerase of *M. tuberculosis* tends to insert G instead of A or C opposite 8-oxoG resulting in 8-oxoG:G mispair [18]. In this case, the activity of MtuFpg1 on 8-oxoG:G would lead to a G to C transition. Although the 8-oxoG:A mispair may occur less frequently in *M. tuberculosis* than in *E. coli*, when A is inserted opposite 8-oxoG, its mutagenic effect could be efficiently prevented by MutY. Recently, mycobacterial MutY has been shown to recognize A, G and T but not C opposite 8-oxoG, unlike EcoMutY which only sees 8-oxoG:A [71]. Thus MtuMutY can remove G from the 8-oxoG mispair

formed during *M. tuberculosis* replication past 8-oxoG. Taken together, the mycobacterial proteins have adapted to the high G-C content of their genome.

4.2. Pseudogenization may facilitate the pathogenesis of *M. tuberculosis*

Although Sidorenko *et al.* have shown that MtuFpg2 slightly increased (about 1.5 fold) the mutation frequency of *E. coli* CC104 *fpg mutY* strain [22], our biochemical data suggest that the gene encoding MtuFpg2 is most likely a pseudogene. During prolonged intracellular survival, mycobacteria tend to eliminate their important pathogenic genes, thereby reducing virulence and facilitating endosymbiosis [72]. Some other nonpathogenic mycobacteria, such as *M. smegmatis* and *Mycobacterium avium*, harbor a full-length Fpg2 homolog. We thus propose that the redundant Fpg enzymes may have at some point contributed to the virulence of the pathogen in the nonhost environment. DNA glycosylase assays on these full-length Fpg2 homologs should elucidate the original biochemical activity of MtuFpg2. The most parsimonious assumption for the activity of Fpg2 is that it is similar to those of other Fpg proteins. Nevertheless, the [26,29] presence of redundant Fpg proteins raises the possibility that Fpg2 has assumed some new function or mode of operation in at least a few species [73]. An extreme case of reductive evolution is *M. leprae* [74]. Surprisingly, although more than half of its genes exist as pseudogenes, *M. leprae* maintains the full-length orthologs of MtuFpg1 and MtuNth, which again indicates the important physiological role of these DNA glycosylases.

4.3. Recognizing single-stranded DNA is a property of the Fpg/Nei family members

EcoNei, MtuNei1 and NEIL1 are all capable of removing oxidized pyrimidines not only from double-stranded oligodeoxynucleotides but also from single-stranded oligodeoxynucleotides (Figs. 3 and 4 and [26,29,61,75]). The two prokaryotic Nei proteins studied are also functional on bubble structures and 3' forked structure, as are their eukaryotic homologs (Fig. 5 and [26,29]). In addition, both MtuFpg1 and EcoFpg (Fig. 4A and [48,76]) exhibit lyase activity on single-stranded substrates and EcoFpg recognizes a single-stranded substrate containing 8-oxoG (Fig. 4A and [62,63]). Taken together with the recent observations on Nei orthologs from Mimivirus [64] as well as Fpg homologs derived from *Candida albicans* and *Arabidopsis thaliana* [34], the data suggest that recognizing lesions in single-stranded DNA as well as other DNA structures containing single-stranded regions is not exclusively a function of the human/mammalian NEILs [26,29,61,75,77], but rather a characteristic of the Fpg/Nei family members in general. Since transient single-stranded DNA is formed during DNA replication and transcription, the replication-associated DNA repair functions proposed for mammalian cells [29] may also occur in prokaryotes, lower eukaryotes and plants.

4.4. MtuNei1 and MtuNth exhibit stringent stereospecificities and are catalytically complementary in repairing Tg

We observed that MtuNth strongly prefers the (5S) *cis-trans* pair of Tg, while MtuNei1 removes the (5R) *cis-trans* pair much more efficiently (Fig. 6). The two enzymes are catalytically complementary in repairing Tg and more stereoselective compared to their *E. coli* homologs. Our observation on the stereospecificity of EcoNth and EcoNei is consistent with what has been reported [66,67]. Interestingly, mammalian NTH proteins exhibit opposite stereospecificities compared to their prokaryotic homologs and prefer the (5R) *cis-trans* pair of Tg [66,67,78,79]. Moreover, although human NEIL1 prefers the (5R) isomers [66,78] as EcoNei does, mouse NEIL1 prefers the (5S) isomers [67].

4.5. MtuNei2 is under the control of a RecA-independent promoter

Nei proteins are missing from the majority of eubacterial genomes sequenced thus far except for actinobacteria. The common ancestor of all the actinomycetes had two Fpg and two Nei

proteins [73]. The gene encoding MtuNei2 (*Rv3297*) is predicted to be co-transcribed with a potential ATP-dependent helicase gene *lhr* (long helicase-related), which is immediately upstream to it (<http://www.microbesonline.org/operons/gnc83332.html>). The putative operon is under the control of a recently identified RecA-independent promoter (RecA-NDp) and could be induced through a novel RecA-independent mechanism [14,15]. Even though disrupting *lhr* in *E. coli* did not show any effect on cell growth or enhanced sensitivities towards UV irradiation and H₂O₂ treatment [80], the potential role of *lhr* during infection and in the DNA damage response has been underscored by the fact that the expression of *lhr* in *M. tuberculosis* could be upregulated by UV irradiation and mitomycin C treatment and induced in the macrophage environment [81,82]. Therefore, it is possible that the interaction between Lhr and MtuNei2 may be crucial for recruiting MtuNei2 onto damaged DNA and stimulating the activity of MtuNei2 during DNA repair. Furthermore, the *lhr/nei2* operon as well as an upstream promoter similar to *M. tuberculosis* RecA-NDp is present in most if not all the actinobacteria, which strongly suggests that the operon is under selection. Although the biological function of MtuNei2 appears to be redundant to that of MtuNei1 (Fig. 7 and Table S2), its activity is regulated differently and may be inducible upon infection or after DNA damage. Thus rather than possessing a complementary activity, MtuNei2 may act as a backup for MtuNei1 under stressful conditions or function during a different life stage of *M. tuberculosis*.

4.6. Mycobacterial enzymes are catalytically slower than their *E. coli* homologs

Our kinetic data indicate that the overall rate constants for the *M. tuberculosis* enzymes, particularly MtuFpg1 and MtuNei1 are lower than those of their *E. coli* counterparts. This is possibly due to the slower growth rate of the pathogen, which might obviate the need for catalytically efficient glycosylases. Moreover, even though these mycobacterial enzymes exhibit significant sequence similarity to their *E. coli* homologs, structural differences, as yet undefined, may account not only for lower catalytic rates but also for the altered substrate preferences of these enzymes.

5. Conclusions

Mycobacterial DNA repair mechanisms may contribute to its survival and persistent infection. The genome of *M. tuberculosis* is highly G/C-rich, which renders the pathogen more susceptible to oxidative DNA damage. It turns out that *M. tuberculosis* is well equipped to deal with this potential problem harboring at least three Fpg/Nei members and one Nth homolog as well as other BER enzymes. Here we overexpressed, purified and characterized MtuFpg1, MtuFpg2, MtuNei1 and MtuNth *in vitro* and evaluated the biological role of MtuNei2 *in vivo*. We have shown that the substrate specificities of these DNA glycosylases are overlapping but less redundant compared to their *E. coli* counterparts. Our data provide direct biochemical evidence for the importance of these mycobacterial DNA glycosylases in DNA repair and suggest that BER is an essential process to maintain the genomic stability of *M. tuberculosis*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

References

- Wallace SS. Biological consequences of free radical-damaged DNA bases. *Free Radic Biol Med* 2002;33:1–14. [PubMed: 12086677]
- David SS, O'Shea VL, Kundu S. Base-excision repair of oxidative DNA damage. *Nature* 2007;447:941–950. [PubMed: 17581577]
- Lindahl T, Barnes DE. Repair of endogenous DNA damage. *Cold Spring Harb Symp Quant Biol* 2000;65:127–133. [PubMed: 12760027]
- Wallace SS, Bandaru V, Kathe SD, Bond JP. The enigma of endonuclease VIII. *DNA Repair (Amst)* 2003;2:441–453. [PubMed: 12713806]
- Wilson DM 3rd, Sofinowski TM, McNeill DR. Repair mechanisms for oxidative DNA damage. *Front Biosci* 2003;8:d963–981. [PubMed: 12700077]
- Krokan HE, Nilsen H, Skorpen F, Otterlei M, Slupphaug G. Base excision repair of DNA in mammalian cells. *FEBS Lett* 2000;476:73–77. [PubMed: 10878254]
- Aravind L, Walker DR, Koonin EV. Conserved domains in DNA repair proteins and evolution of repair systems. *Nucleic Acids Res* 1999;27:1223–1242. [PubMed: 9973609]
- Eisen JA, Hanawalt PC. A phylogenomic study of DNA repair genes, proteins, and processes. *Mutat Res* 1999;435:171–213. [PubMed: 10606811]
- Dizdaroglu M. Base-excision repair of oxidative DNA damage by DNA glycosylases. *Mutat Res* 2005;591:45–59. [PubMed: 16054172]
- O'Rourke EJ, Chevalier C, Pinto AV, Thiberge JM, Ielpi L, Labigne A, Radicella JP. Pathogen DNA as target for host-generated oxidative stress: role for repair of bacterial DNA damage in *Helicobacter pylori* colonization. *Proc Natl Acad Sci U S A* 2003;100:2789–2794. [PubMed: 12601164]
- Suvarnapunya AE, Lagasse HA, Stein MA. The role of DNA base excision repair in the pathogenesis of *Salmonella enterica* serovar Typhimurium. *Mol Microbiol* 2003;48:549–559. [PubMed: 12675811]
- Mizrahi V, Andersen SJ. DNA repair in *Mycobacterium tuberculosis*. What have we learnt from the genome sequence? *Mol Microbiol* 1998;29:1331–1339. [PubMed: 9781872]
- Dos Vultos T, Mestre O, Tonjum T, Gicquel B. DNA repair in *Mycobacterium tuberculosis* revisited. *FEMS Microbiol Rev* 2009;33:471–487. [PubMed: 19385996]
- Gamulin V, Cetkovic H, Ahel I. Identification of a promoter motif regulating the major DNA damage response mechanism of *Mycobacterium tuberculosis*. *FEMS Microbiol Lett* 2004;238:57–63. [PubMed: 15336403]
- Rand L, Hinds J, Springer B, Sander P, Buxton RS, Davis EO. The majority of inducible DNA repair genes in *Mycobacterium tuberculosis* are induced independently of RecA. *Mol Microbiol* 2003;50:1031–1042. [PubMed: 14617159]
- Darwin KH, Nathan CF. Role for nucleotide excision repair in virulence of *Mycobacterium tuberculosis*. *Infect Immun* 2005;73:4581–4587. [PubMed: 16040969]
- Venkatesh J, Kumar P, Krishna PS, Manjunath R, Varshney U. Importance of uracil DNA glycosylase in *Pseudomonas aeruginosa* and *Mycobacterium smegmatis*, G+C-rich bacteria, in mutation prevention, tolerance to acidified nitrite, and endurance in mouse macrophages. *J Biol Chem* 2003;278:24350–24358. [PubMed: 12679366]
- Jain R, Kumar P, Varshney U. A distinct role of formamidopyrimidine DNA glycosylase (MutM) in down-regulation of accumulation of G, C mutations and protection against oxidative stress in mycobacteria. *DNA Repair (Amst)* 2007;6:1774–1785. [PubMed: 17698424]

19. Sassetti CM, Rubin EJ. Genetic requirements for mycobacterial survival during infection. *Proc Natl Acad Sci U S A* 2003;100:12989–12994. [PubMed: 14569030]
20. Rengarajan J, Bloom BR, Rubin EJ. Genome-wide requirements for *Mycobacterium tuberculosis* adaptation and survival in macrophages. *Proc Natl Acad Sci U S A* 2005;102:8327–8332. [PubMed: 15928073]
21. Cole ST, Brosch R, Parkhill J, Garnier T, Churcher C, Harris D, Gordon SV, Eiglmeier K, Gas S, Barry CE 3rd, Tekaia F, Badcock K, Basham D, Brown D, Chillingworth T, Connor R, Davies R, Devlin K, Feltwell T, Gentles S, Hamlin N, Holroyd S, Hornsby T, Jagels K, Krogh A, McLean J, Moule S, Murphy L, Oliver K, Osborne J, Quail MA, Rajandream MA, Rogers J, Rutter S, Seeger K, Skelton J, Squares R, Squares S, Sulston JE, Taylor K, Whitehead S, Barrell BG. Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* 1998;393:537–544. [PubMed: 9634230]
22. Sidorenko VS, Rot MA, Filipenko ML, Nevinsky GA, Zharkov DO. Novel DNA glycosylases from *Mycobacterium tuberculosis*. *Biochemistry (Mosc)* 2008;73:442–450. [PubMed: 18457574]
23. Olsen I, Balasingham SV, Davidsen T, Debebe E, Rodland EA, van Soolingen D, Kremer K, Alseth I, Tonjum T. Characterization of the major formamidopyrimidine-DNA glycosylase homolog in *Mycobacterium tuberculosis* and its linkage to variable tandem repeats. *FEMS Immunol Med Microbiol* 2009;56:151–161. [PubMed: 19496823]
24. Guo Y, Wallace SS, Bandaru V. A novel bicistronic vector for overexpressing *Mycobacterium tuberculosis* proteins in *Escherichia coli*. *Protein Expr Purif* 2009;65:230–237. [PubMed: 19162193]
25. Bandaru V, Blaisdell JO, Wallace SS. Oxidative DNA glycosylases: recipes from cloning to characterization. *Methods Enzymol* 2006;408:15–33. [PubMed: 16793360]
26. Dou H, Theriot CA, Das A, Hegde ML, Matsumoto Y, Boldogh I, Hazra TK, Bhakat KK, Mitra S. Interaction of the human DNA glycosylase NEIL1 with proliferating cell nuclear antigen. The potential for replication-associated repair of oxidized bases in mammalian genomes. *J Biol Chem* 2008;283:3130–3140. [PubMed: 18032376]
27. Iwai S. Synthesis and thermodynamic studies of oligonucleotides containing the two isomers of thymine glycol. *Chemistry* 2001;7:4343–4351. [PubMed: 11695667]
28. Shimizu T, Manabe K, Yoshikawa S, Kawasaki Y, Iwai S. Preferential formation of (5S,6R)-thymine glycol for oligodeoxyribonucleotide synthesis and analysis of drug binding to thymine glycol-containing DNA. *Nucleic Acids Res* 2006;34:313–321. [PubMed: 16401612]
29. Dou H, Mitra S, Hazra TK. Repair of oxidized bases in DNA bubble structures by human DNA glycosylases NEIL1 and NEIL2. *J Biol Chem* 2003;278:49679–49684. [PubMed: 14522990]
30. Kornysushyna O, Berges AM, Muller JG, Burrows CJ. In vitro nucleotide misinsertion opposite the oxidized guanosine lesions spiroiminodihydantoin and guanidinohydantoin and DNA synthesis past the lesions using *Escherichia coli* DNA polymerase I (Klenow fragment). *Biochemistry* 2002;41:15304–15314. [PubMed: 12484769]
31. Ide H, Kow YW, Wallace SS. Thymine glycols and urea residues in M13 DNA constitute replicative blocks in vitro. *Nucleic Acids Res* 1985;13:8035–8052. [PubMed: 3906566]
32. Blaisdell JO, Wallace SS. Rapid determination of the active fraction of DNA repair glycosylases: a novel fluorescence assay for trapped intermediates. *Nucleic Acids Res* 2007;35:1601–1611. [PubMed: 17289752]
33. Dizdaroglu M, Bauche C, Rodriguez H, Laval J. Novel substrates of *Escherichia coli* nth protein and its kinetics for excision of modified bases from DNA damaged by free radicals. *Biochemistry* 2000;39:5586–5592. [PubMed: 10820032]
34. Kathe SD, Barrantes-Reynolds R, Jaruga P, Newton MR, Burrows CJ, Bandaru V, Dizdaroglu M, Bond JP, Wallace SS. Plant and fungal Fpg homologs are formamidopyrimidine DNA glycosylases but not 8-oxoguanine DNA glycosylases. *DNA Repair (Amst)* 2009;8:643–653. [PubMed: 19217358]
35. Jaruga P, Birincioglu M, Rosenquist TA, Dizdaroglu M. Mouse NEIL1 protein is specific for excision of 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 4,6-diamino-5-formamidopyrimidine from oxidatively damaged DNA. *Biochemistry* 2004;43:15909–15914. [PubMed: 15595846]
36. Blaisdell JO, Hatahet Z, Wallace SS. A novel role for *Escherichia coli* endonuclease VIII in prevention of spontaneous G→T transversions. *J Bacteriol* 1999;181:6396–6402. [PubMed: 10515930]

37. Jiang D, Hatahet Z, Blaisdell JO, Melamede RJ, Wallace SS. Escherichia coli endonuclease VIII: cloning, sequencing, and overexpression of the nei structural gene and characterization of nei and nei nth mutants. *J Bacteriol* 1997;179:3773–3782. [PubMed: 9171429]
38. Tchou J, Bodepudi V, Shibutani S, Antoshechkin I, Miller J, Grollman AP, Johnson F. Substrate specificity of Fpg protein. Recognition and cleavage of oxidatively damaged DNA. *J Biol Chem* 1994;269:15318–15324. [PubMed: 7515054]
39. Leipold MD, Muller JG, Burrows CJ, David SS. Removal of hydantoin products of 8-oxoguanine oxidation by the Escherichia coli DNA repair enzyme, FPG. *Biochemistry* 2000;39:14984–14992. [PubMed: 11101315]
40. Burrows CJ, Muller JG, Kornysheva O, Luo W, Duarte V, Leipold MD, David SS. Structure and potential mutagenicity of new hydantoin products from guanosine and 8-oxo-7,8-dihydroguanine oxidation by transition metals. *Environ Health Perspect* 2002;110(Suppl 5):713–717. [PubMed: 12426118]
41. Henderson PT, Delaney JC, Muller JG, Neeley WL, Tannenbaum SR, Burrows CJ, Essigmann JM. The hydantoin lesions formed from oxidation of 7,8-dihydro-8-oxoguanine are potent sources of replication errors in vivo. *Biochemistry* 2003;42:9257–9262. [PubMed: 12899611]
42. Krishnamurthy N, Muller JG, Burrows CJ, David SS. Unusual structural features of hydantoin lesions translate into efficient recognition by Escherichia coli Fpg. *Biochemistry* 2007;46:9355–9365. [PubMed: 17655276]
43. Hazra TK, Muller JG, Manuel RC, Burrows CJ, Lloyd RS, Mitra S. Repair of hydantoins, one electron oxidation product of 8-oxoguanine, by DNA glycosylases of Escherichia coli. *Nucleic Acids Res* 2001;29:1967–1974. [PubMed: 11328881]
44. Zharkov DO, Rieger RA, Iden CR, Grollman AP. NH₂-terminal proline acts as a nucleophile in the glycosylase/AP-lyase reaction catalyzed by Escherichia coli formamidopyrimidine-DNA glycosylase (Fpg) protein. *J Biol Chem* 1997;272:5335–5341. [PubMed: 9030608]
45. Lavrakhin OV, Lloyd RS. Involvement of phylogenetically conserved acidic amino acid residues in catalysis by an oxidative DNA damage enzyme formamidopyrimidine glycosylase. *Biochemistry* 2000;39:15266–15271. [PubMed: 11106507]
46. Gilboa R, Zharkov DO, Golan G, Fernandes AS, Gerchman SE, Matz E, Kycia JH, Grollman AP, Shoham G. Structure of formamidopyrimidine-DNA glycosylase covalently complexed to DNA. *J Biol Chem* 2002;277:19811–19816. [PubMed: 11912217]
47. Zharkov DO, Golan G, Gilboa R, Fernandes AS, Gerchman SE, Kycia JH, Rieger RA, Grollman AP, Shoham G. Structural analysis of an Escherichia coli endonuclease VIII covalent reaction intermediate. *Embo J* 2002;21:789–800. [PubMed: 11847126]
48. Castaing B, Boiteux S, Zelwer C. DNA containing a chemically reduced apurinic site is a high affinity ligand for the E. coli formamidopyrimidine-DNA glycosylase. *Nucleic Acids Res* 1992;20:389–394. [PubMed: 1741272]
49. O'Connor TR, Graves RJ, de Murcia G, Castaing B, Laval J. Fpg protein of Escherichia coli is a zinc finger protein whose cysteine residues have a structural and/or functional role. *J Biol Chem* 1993;268:9063–9070. [PubMed: 8473347]
50. Tchou J, Michaels ML, Miller JH, Grollman AP. Function of the zinc finger in Escherichia coli Fpg protein. *J Biol Chem* 1993;268:26738–26744. [PubMed: 8253809]
51. Sugahara M, Mikawa T, Kumasaka T, Yamamoto M, Kato R, Fukuyama K, Inoue Y, Kuramitsu S. Crystal structure of a repair enzyme of oxidatively damaged DNA, MutM (Fpg), from an extreme thermophile, *Thermus thermophilus* HB8. *Embo J* 2000;19:3857–3869. [PubMed: 10921868]
52. Fromme JC, Verdine GL. Structural insights into lesion recognition and repair by the bacterial 8-oxoguanine DNA glycosylase MutM. *Nat Struct Biol* 2002;9:544–552. [PubMed: 12055620]
53. Melamede RJ, Hatahet Z, Kow YW, Ide H, Wallace SS. Isolation and characterization of endonuclease VIII from Escherichia coli. *Biochemistry* 1994;33:1255–1264. [PubMed: 8110759]
54. Jiang D, Hatahet Z, Melamede RJ, Kow YW, Wallace SS. Characterization of Escherichia coli endonuclease VIII. *J Biol Chem* 1997;272:32230–32239. [PubMed: 9405426]
55. Bandaru V, Sunkara S, Wallace SS, Bond JP. A novel human DNA glycosylase that removes oxidative DNA damage and is homologous to Escherichia coli endonuclease VIII. *DNA Repair (Amst)* 2002;1:517–529. [PubMed: 12509226]

56. Hazra TK, Izumi T, Boldogh I, Imhoff B, Kow YW, Jaruga P, Dizdaroglu M, Mitra S. Identification and characterization of a human DNA glycosylase for repair of modified bases in oxidatively damaged DNA. *Proc Natl Acad Sci U S A* 2002;99:3523–3528. [PubMed: 11904416]
57. Hazra TK, Kow YW, Hatahet Z, Imhoff B, Boldogh I, Mokkapati SK, Mitra S, Izumi T. Identification and characterization of a novel human DNA glycosylase for repair of cytosine-derived lesions. *J Biol Chem* 2002;277:30417–30420. [PubMed: 12097317]
58. Morland I, Rolseth V, Luna L, Rognes T, Bjoras M, Seeberg E. Human DNA glycosylases of the bacterial Fpg/MutM superfamily: an alternative pathway for the repair of 8-oxoguanine and other oxidation products in DNA. *Nucleic Acids Res* 2002;30:4926–4936. [PubMed: 12433996]
59. Hailer MK, Slade PG, Martin BD, Sugden KD. Nei deficient *Escherichia coli* are sensitive to chromate and accumulate the oxidized guanine lesion spiroiminodihydantoin. *Chem Res Toxicol* 2005;18:1378–1383. [PubMed: 16167829]
60. Krishnamurthy N, Zhao X, Burrows CJ, David SS. Superior removal of hydantoin lesions relative to other oxidized bases by the human DNA glycosylase hNEIL1. *Biochemistry* 2008;47:7137–7146. [PubMed: 18543945]
61. Hailer MK, Slade PG, Martin BD, Rosenquist TA, Sugden KD. Recognition of the oxidized lesions spiroiminodihydantoin and guanidinohydantoin in DNA by the mammalian base excision repair glycosylases NEIL1 and NEIL2. *DNA Repair (Amst)* 2005;4:41–50. [PubMed: 15533836]
62. Ishchenko AA, Bulychev NV, Maksakova GA, Johnson F, Nevinsky GA. Single-stranded oligodeoxyribonucleotides are substrates of Fpg protein from *Escherichia coli*. *IUBMB Life* 1999;48:613–618. [PubMed: 10683766]
63. Ishchenko AA, Koval VV, Fedorova OS, Douglas KT, Nevinsky GA. Structural requirements of double and single stranded DNA substrates and inhibitors, including a photoaffinity label, of Fpg protein from *Escherichia coli*. *J Biomol Struct Dyn* 1999;17:301–310. [PubMed: 10563580]
64. Bandaru V, Zhao X, Newton MR, Burrows CJ, Wallace SS. Human endonuclease VIII-like (NEIL) proteins in the giant DNA Mimivirus. *DNA Repair (Amst)* 2007;6:1629–1641. [PubMed: 17627905]
65. Teebor G, Cummings A, Frenkel K, Shaw A, Voituriez L, Cadet J. Quantitative measurement of the diastereoisomers of cis thymidine glycol in gamma-irradiated DNA. *Free Radic Res Commun* 1987;2:303–309. [PubMed: 3504811]
66. Katafuchi A, Nakano T, Masaoka A, Terato H, Iwai S, Hanaoka F, Ide H. Differential specificity of human and *Escherichia coli* endonuclease III and VIII homologues for oxidative base lesions. *J Biol Chem* 2004;279:14464–14471. [PubMed: 14734554]
67. Miller H, Fernandes AS, Zaika E, McTigue MM, Torres MC, Wente M, Iden CR, Grollman AP. Stereoselective excision of thymine glycol from oxidatively damaged DNA. *Nucleic Acids Res* 2004;32:338–345. [PubMed: 14726482]
68. Castaing B, Geiger A, Seliger H, Nehls P, Laval J, Zelwer C, Boiteux S. Cleavage and binding of a DNA fragment containing a single 8-oxoguanine by wild type and mutant FPG proteins. *Nucleic Acids Res* 1993;21:2899–2905. [PubMed: 8332499]
69. Leipold MD, Muller JG, Burrows CJ, David SS. Removal of hydantoin products of 8-oxoguanine oxidation by the *Escherichia coli* DNA repair enzyme, FPG. *Biochemistry (Mosc)* 2000;39:14984–14992.
70. Krishnamurthy N, Muller JG, Burrows CJ, David SS. Unusual structural features of hydantoin lesions translate into efficient recognition by *Escherichia coli* Fpg. *Biochemistry (Mosc)* 2007;46:9355–9365.
71. Kurthkoti K, Srinath T, Kumar P, Malshetty VS, Sang PB, Jain R, Manjunath R, Varshney U. A distinct physiological role of MutY in mutation prevention in mycobacteria. *Microbiology*. 2009 in press.
72. Gericke GS. Is there an emerging endosymbiotic relationship between mycobacteria and the human host based on horizontal transfer of genetic sequences? *Med Hypotheses* 2006;67:1419–1428. [PubMed: 16919888]
73. Pumo, D.; Barrantes-Reynolds, R.; Kathe, S.; Wallace, S.; Bond, J. Genetic and Epigenetic Regulation of Genome Stability and Transgenerational Response to Stress. Nova Science Publishers, Inc; Hauppauge, New York: 2009. Evolution of the Fpg/Nei family of DNA glycosylases. in press

74. Cole ST, Eglmeier K, Parkhill J, James KD, Thomson NR, Wheeler PR, Honore N, Garnier T, Churcher C, Harris D, Mungall K, Basham D, Brown D, Chillingworth T, Connor R, Davies RM, Devlin K, Duthoy S, Feltwell T, Fraser A, Hamlin N, Holroyd S, Hornsby T, Jagels K, Lacroix C, Maclean J, Moule S, Murphy L, Oliver K, Quail MA, Rajandream MA, Rutherford KM, Rutter S, Seeger K, Simon S, Simmonds M, Skelton J, Squares R, Squares S, Stevens K, Taylor K, Whitehead S, Woodward JR, Barrell BG. Massive gene decay in the leprosy bacillus. *Nature* 2001;409:1007–1011. [PubMed: 11234002]
75. Zhang QM, Yonekura S, Takao M, Yasui A, Sugiyama H, Yonei S. DNA glycosylase activities for thymine residues oxidized in the methyl group are functions of the hNEIL1 and hNTH1 enzymes in human cells. *DNA Repair (Amst)* 2005;4:71–79. [PubMed: 15533839]
76. Neto JB, Gentil A, Cabral RE, Sarasin A. Mutation spectrum of heat-induced abasic sites on a single-stranded shuttle vector replicated in mammalian cells. *J Biol Chem* 1992;267:19718–19723. [PubMed: 1527092]
77. Takao M, Kanno S, Kobayashi K, Zhang QM, Yonei S, van der Horst GT, Yasui A. A back-up glycosylase in Nth1 knock-out mice is a functional Nei (endonuclease VIII) homologue. *J Biol Chem* 2002;277:42205–42213. [PubMed: 12200441]
78. Ocampo-Hafalla MT, Altamirano A, Basu AK, Chan MK, Ocampo JE, Cummings A Jr, Boorstein RJ, Cunningham RP, Teebor GW. Repair of thymine glycol by hNth1 and hNei1 is modulated by base pairing and cis-trans epimerization. *DNA Repair (Amst)* 2006;5:444–454. [PubMed: 16446124]
79. McTigue MM, Rieger RA, Rosenquist TA, Iden CR, De Los Santos CR. Stereoselective excision of thymine glycol lesions by mammalian cell extracts. *DNA Repair (Amst)* 2004;3:313–322. [PubMed: 15177046]
80. Reuven NB, Koonin EV, Rudd KE, Deutscher MP. The gene for the longest known Escherichia coli protein is a member of helicase superfamily II. *J Bacteriol* 1995;177:5393–5400. [PubMed: 7559321]
81. Boshoff HI, Reed MB, Barry CE 3rd, Mizrahi V. DnaE2 polymerase contributes to in vivo survival and the emergence of drug resistance in Mycobacterium tuberculosis. *Cell* 2003;113:183–193. [PubMed: 12705867]
82. Schnappinger D, Ehrt S, Voskuil MI, Liu Y, Mangan JA, Monahan IM, Dolganov G, Efron B, Butcher PD, Nathan C, Schoolnik GK. Transcriptional Adaptation of Mycobacterium tuberculosis within Macrophages: Insights into the Phagosomal Environment. *J Exp Med* 2003;198:693–704. [PubMed: 12953091]

Fig. 1. DNA glycosylase/lyase activity of MtuFpg1 on double-stranded DNA substrates. Labeled substrate, 15 nM, was incubated with either no enzyme (–), 15 nM EcoFpg or increasing amounts of MtuFpg1 (1.5 nM, 15 nM or 150 nM).

Fig. 2. Enzymatic activities of MtuFpg1 (●) and EcoFpg (○) on 8-oxoG:A. Labeled 8-oxoG:A, 15 nM, was incubated with increasing amounts of either MtuFpg1 or EcoFpg at 37 °C for 30 min. The data represent the means of three independent experiments, the uncertainties are standard deviations.

Fig. 3.

(A) DNA glycosylase/lyase activities of MtuNth and MtuNei1 on double-stranded DNA substrates. Labeled substrate, 15 nM, was incubated with no enzyme (-), 15 nM EcoNth, EcoNei or NEIL1, or increasing amounts of MtuNth or MtuNei1 (1.5 nM, 15 nM or 150 nM). (B) Uracil DNA glycosylase activity of MtuNei1. Double-stranded DNA substrate containing uracil, 15 nM, was incubated with no enzyme (-), 15 nM EcoFpg, MtuFpg1, EcoNth, MtuNth, EcoNei or NEIL1, or increasing amounts of MtuNei1 (1.5 nM, 15 nM or 150 nM).

(B)

Fig. 4.

DNA glycosylase/lyase activity of MtuNei1 on single-stranded DNA substrates. (A) Labeled substrate, 15 nM, was incubated with no enzyme (–), 15 nM EcoFpg, MtuFpg, EcoNth, MtuNth, EcoNei or NEIL1, or MtuNei1. (B) The fraction cleaved was calculated for the reactions with 15 nM enzyme. The data represent the means of three independent experiments and the error bars indicate the standard deviations.

Fig. 5. Activity of MtuNei1 on 5-OHU-containing (A) DNA bubble structures with 5, 11 or 19 unpaired bases and (B) 3' forked structure. Labeled substrate, 15 nM, was incubated with 15 nM EcoNei, NEIL1 or MtuNei1. Cleaved fractions were calculated for each reaction. The double-stranded and single-stranded 51-mer substrates containing 5-OHU were used as controls for the reactions with the bubble structures. The double-stranded and single-stranded 35-mer substrates containing 5-OHU were controls for the reactions with the 3' forked structure. The data represent the means of three independent experiments, the uncertainties are standard deviations.

Fig. 6. Stereospecificity of MtuNth and MtuNei1 on thymine glycol. Double-stranded oligodeoxynucleotide substrates, 15 nM, containing either 5R ((5R,6S), (5R,6R)) or 5S ((5S,6R), (5S,6S)) Tg isomers were incubated with either no enzyme (–) or increasing amounts of MtuNth, EcoNth, MtuNei1 and EcoNei (1.5 nM, 15 nM or 150 nM).

Fig. 7.

Spontaneous mutation frequencies to rifampin resistance in *E. coli* wild type (W.T.), *fpg mutY nei* triple mutant, *nei nth* double mutant as well as the mutant strains expressing either EcoNei, MtuNei1 or MtuNei2. Mid-log-phase cultures were plated on LB agar with or without 100 µg/ml of rifampin. Mutation frequencies per 10⁸ cells were calculated. The data shown are the averages of the three or four independent experiments. The error bars indicate the standard deviations. * - significant difference ($p < 0.05$ by the student's *t* test) compared to the mutation frequency of the *fpg mutY nei* triple mutant or the *nei nth* double mutant.

Table 1

Kinetic parameters: k_{obs} (min^{-1}) and $(k_{\text{cat}}/K_m)^{\text{app}}$ ($\text{min}^{-1}\text{nM}^{-1}$) of MtuFpg1, MtuNei1 and MtuNth as well as their *E. coli* homologs on double-stranded oligodeoxynucleotide substrates.

Substrates	MtuFpg1		EcoFpg		MtuNei1		EcoNei		MtuNth		EcoNth	
	k_{obs}	$(k_{\text{cat}}/K_m)^{\text{app}}$	k_{obs}	$(k_{\text{cat}}/K_m)^{\text{app}}$	k_{obs}	$(k_{\text{cat}}/K_m)^{\text{app}}$	k_{obs}	$(k_{\text{cat}}/K_m)^{\text{app}}$	k_{obs}	$(k_{\text{cat}}/K_m)^{\text{app}}$	k_{obs}	$(k_{\text{cat}}/K_m)^{\text{app}}$
8-oxoG:C	1.50	0.029	15.1	0.012	ND	ND	ND	ND				
Sp:C	4.58	0.30	34.2	5.05	0.46	0.065	13.0	1.39				
Tg:A	ND	ND	0.74	0.012	5.55/0.46	0.010	82.0/4.90	3.30	0.32/19.0	0.044	16.0/15.2	1.18
Tg:G	0.06	0.00092	0.69	0.16	8.01/0.59	0.025	260/23.4	8.84	0.027/22.9	0.033	22.6/283.9	0.41
DHU:G	0.14	0.0041	6.58	0.30	1.43	0.028	150	2.11	14.1	0.37	46.4	0.38
5-OHC:G					0.97	0.0090	63.3	0.30	76.7	0.083	25.8	0.18

The amount of each enzyme was corrected for the fraction of active enzyme. The reactions were terminated at appropriate time points with formamide stop loading buffer. The k_{obs} and the $(k_{\text{cat}}/K_m)^{\text{app}}$ values were solved under single-turnover conditions and multiple-turnover conditions respectively, as described in Materials and Methods. Data were analyzed with Prism 4 (GraphPad Software, Inc. La Jolla, CA). (ND – not detectable)

The activities of mycobacterial DNA glycosylases as well as their *E. coli* homologs on γ -irradiated DNA as measured by GC/MS.

Table 2

	MtuFpgI	EcoFpg	MtuNeiI	EcoNei	MtuNth	EcoNth
MeHyd	0.9±0.5	2.4±1.2	18±0.5	23±0.5	20±0.9	27±0.7
5-OHC			4.1±0.3	55±3.2	160±9	246±3
Tg			10±1.4	51±1.2	6.4±0.5	73±1.8
FapyA	84±1.5	202±6	20±0.8	148±0.6	66±2.6	118±1
8-oxoA	2.6±0.6	46±1.8			14±0.5	21±0.7
FapyG	387±4	683±22	16±0.9	15±1.2	18±1.1	12±0.6
8-oxoG	246±6	625±19				
5-OHU			8.9±1.7	11±1.6	108±6.8	199±15

The same amounts of active enzymes were used in each reaction. Numbers represent released damaged bases per 10⁶ total DNA bases after 1 hour incubation with γ -irradiated DNA in substrate excess. The data represent the means of three independent experiments. The uncertainties are standard deviations.