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Novel DNA Glycosylases from *Mycobacterium tuberculosis*

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Abstract—Oxidized bases are removed from DNA of *Escherichia coli* by enzymes formamidopyrimidine DNA glycosylase (*Eco*-Fpg) and endonuclease VIII (*Eco*-Nei) of the same structural family Fpg/Nei. New homologs of these enzymes not characterized earlier have been found in genomes of Actinobacteria. We have cloned and expressed two paralogs (*Mtu*-Nei2 and *Mtu*-Fpg2) from 36KAZ and KHA94 isolates of *Mycobacterium tuberculosis* and studied their ability to participate in DNA repair. Under heterologous expression in *E. coli*, *Mtu*-Nei2 decreased the rate of spontaneous mutagenesis in the *rpoB* gene, whereas *Mtu*-Fpg2 moderately increased it, possibly due to absence of residues crucially important for catalysis in this protein. *Mtu*-Nei2 was highly active toward double-stranded DNA substrates containing dihydrouracil residues and apurine-apyrimidine sites and was less efficient in cleavage of substrates containing 8-oxoguanine and uracil residues. These lesions, as well as 8-oxoadenine residues, were also recognized and removed by the enzyme from single-stranded DNA. Fpg and Nei homologs from *M. tuberculosis* can play an important role in protection of bacteria against genotoxic stress caused by oxidative burst in macrophages.

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DNA is constantly exposed to various chemical and physical agents. In particular, DNA is affected by reactive oxygen species generated by ionizing radiation and in the course of cell metabolism [1]. Damage to DNA bases can lead to mutations or cell death [2]. To prevent this, cells evolved a system of DNA repair. Oxidative lesions are mainly eliminated from DNA by the base excision repair (BER) system, with the central role played by DNA-*N*-glycosylases hydrolyzing the *N*-glycosidic bond of the damaged base and thus excising it from the DNA [3-6]. Thus, oxidized purines and pyrimidines in *Escherichia coli* cells are repaired by enzymes formamidopyrimidine DNA glycosylase (Fpg, which uses oxidized purines as the main substrates) and endonuclease VIII (Nei, which uses oxidized pyrimidines as the main substrates) of the same structural family and also by the non-homologous endonuclease III (which uses oxidized pyrimidines as the main substrates) [3, 7]. All these enzymes are bifunctional glycosylases capable not only of excising a damaged base but also of cleaving DNA at the produced apurine-apyrimidine (AP) site.

Abbreviations: AP) apurine-apyrimidine; BER) base excision repair; DHU) 5,6-dihydrouracil; ODN) oligodeoxyribonucleotide; 8-oxoG) 7,8-dihydro-8-oxoguanine.

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Oxidative stress plays a very important role in the interactions of the human body with pathogenic bacteria. Presenting macrophages with bacteria induces some genes whose products are responsible for generation of reactive oxygen and nitrogen species, the so-called "oxidative burst" [8]. Damage to infectious microorganisms due to oxidative stress is especially important for innate immune response [9]. Nevertheless, an essential part of the life cycle of some pathogens, including the tuberculosis agent *Mycobacterium tuberculosis*, occurs inside macrophages or in their vicinity [10].

It is possible that DNA repair can provide a mechanism for the successful adaptation of mycobacteria to the conditions of chronic oxidative stress. However, the BER system in pathogenic bacteria is studied insufficiently. For instance, only uracil DNA glycosylase (Ung) of mycobacteria has been characterized at the biochemical and cellular levels [11-15]. Up to now, DNA glycosylases responsible for repair of oxidative DNA lesions were not studied in mycobacteria. Genomic sequencing has shown that these organisms contain proteins homologous to Fpg and Nei from *E. coli* [16-18], but their possible functions and substrate specificities are currently unknown.

In the present work we have cloned the genes from *M. tuberculosis* which encode the proteins *Mtu*-Nei2 and

Mtu-Fpg2, determined their ability to complement the phenotype in bacteria deficient in the genes of the system responsible for repair of DNA oxidative lesions, and characterized the substrate specificity of the partially purified protein *Mtu*-Nei2.

MATERIALS AND METHODS

Oligodeoxyribonucleotides, enzymes, and bacterial strains. For cloning and sequencing of fragments of the genes *mtu-fpg2* and *mtu-nei2*, we used oligodeoxyribonucleotides (ODNs) with the following sequences:

Nei2-fwd, 5′-d(CGAGACCATATGCCTGAGGGGCATACGCTGCATCG)-3′;

Nei2-rev, 5′-d(CGAGACGGATCCTCAGGTCTGACAGACCGGGC)-3′;

Fpg2-fwd, 5′-d(CGAGACCCATGGCCGGGACGCCGCAGCC)-3′;

Fpg2-rev, 5′-d(CGAGACGGATCCCTACTTGAGCAGCCGCG)-3′;

pET-fwd, 5′-d(TAATACGACTCACTATAGG)-3′;

pET-rev, 5′-d(GCTAGTTATTGCTCAGCGG)-3′.

To determine the substrate specificity of *Mtu*-Nei2, the following ODNs were used:

OG23, 5′-d(CTCTCCCTTCXCTCCTTTCCTCT)-3′ (X = 8-oxoG);

OA23, 5′-d(CTCTCCCTTCXCTCCTTTCCTCT)-3′ (X = 8-oxoA);

U23, 5′-d(CTCTCCCTTCXCTCCTTTCCTCT)-3′ (X = U);

DHU23, 5′-d(CTCTCCCTTCXCTCCTTTCCTCT)-3′ (X = DHU);

C23, 5′-d(AGAGGAAAGGAGCGAAGGGAGAG)-3′;

T23, 5′-d(AGAGGAAAGGAGTGAAGGGAGAG)-3′;

A23, 5′-d(AGAGGAAAGGAGAGAAGGGAGAG)-3′;

G23, 5′-d(AGAGGAAAGGAGGGAAGGGAGAG)-3′.

The ODNs were synthesized by the phosphoramidite method on a model 394 automatic solid phase synthesizer (Applied Biosystems, USA) and purified by reversed-phase high-pressure liquid chromatography on a PRP-1 column (Hamilton, USA). Double-stranded ODN substrates were prepared by annealing equimolar quantities of one of the ^{32}P-labeled modified strands (OG23, OA23, U23, or DHU23) and of one of the unlabeled complementary strands (C23, T23, A23, or G23).

The enzymes Fpg and Nei were isolated from *E. coli* as described in [19, 20]. In addition, the following enzymes were used: phage T4 polynucleotide kinase, phage T4 DNA ligase, and Ung from *E. coli* (New England Biolabs, USA); RNase A and proteinase K (Sigma, USA); DNA polymerase Pfu, alkaline phosphatase from calf thymus, and restriction endonucleases *Bam*HI, *Fau*NDI, and *Bsp*19I (SibEnzyme, Russia).

The following *E. coli* strains were used: DH5α (*supE*44 Δ*lac*U169 (ϕ80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1), BL21(DE3)RIL (F$^-$ *ompT* *hsdS$_B$*($r_B^- m_B^-$) *gal dcm* (λ*c*I*ts*857 *ind*1 *S*am7 *nin5 lac*UV5-T7 gene *1*) pRIL) (both strains were from Stratagene, USA); SW2-38 (Hfr *KL16 PO-45*:[*lysA*(*61*)-*serA*(*62*)]/*thi relA spoT1 nth*::*Kan* Δ*nei*::*Cm*) (kindly provided by Prof. S. Wallace, University of Vermont, USA), CC104 (F′(*lacproB lacI*378 *lacZ*503) *ara* Δ(*lacproB*)$_{XIII}$ *rpsL*), and CC104 *fpg mutY* (*CC104 fpg*::*mini-Tet mutY*::*Kan*) (a kind gift from Prof. J. Miller, University of California, USA).

Cloning the *mtu-nei2* and *mtu-fpg2* genes from *M. tuberculosis*. Sequences homologous to the *E. coli* proteins Fpg and Nei were detected during the search in translated genomic sequences of H37Ra, H37Rv, F11, and CDC1551 *M. tuberculosis* strains in the GenBank database with a BLAST algorithm [21]. Based on the corresponding DNA sequences, primers for PCR were designed; genomic DNA of the 36KAZ and KHA94 isolates from *M. tuberculosis* was used as templates for amplification of the sequences *mtu-nei2* and *mtu-fpg2*, respectively. The reaction mixture for PCR contained 300 μM oligonucleotide primers (Nei2-fwd and Nei2-rev for *mtu-nei2*, Fpg2-fwd and Fpg2-rev for *mtu-fpg2*), 2 ng of DNA template, 200 μM dNTP, 67 mM Tris-HCl (pH 8.9), 16 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.05% Tween-20, and DNA polymerase Pfu (0.125 activity units per μl). Thirty-five cycles were performed (30 sec at 95°C, 30 sec at 64°C, 1 min at 72°C). The combined and purified PCR fragments from several mixtures (~1 μg) were treated with the necessary restriction endonucleases (*Bsp*19I and *Bam*HI for *mtu-fpg2*, *Fau*NDI and *Bam*HI for *mtu-nei2*) and ligated into plasmids pET-15b (*mtu-fpg2*) and pET-23a (*mtu-nei2*) hydrolyzed by the same enzymes. After the ligation, the mixture was transferred into the DH5α cells by electroporation. The plasmid DNA isolated from colonies grown on a selective medium was analyzed for presence of the insert by treatment with the same restriction endonucleases. The insert-containing clones were sequenced by the Sanger method. In all cases, the sequence coincided with the expected one. Plasmids with

the correct insert were used for transforming *E. coli* BL21(DE3)RIL cells.

Expression and isolation of the Mtu-Nei2 protein. *Escherichia coli* BL21(DE3)RIL cells carrying the pET-23a-*mtu-nei2* plasmid were grown in 5 ml of LB medium supplemented with ampicillin (50 μg/ml) and chloramphenicol (25 μg/ml) at 37°C with intensive shaking for 16-18 h. An inoculate of the overnight culture (500 μl) was introduced into 60 ml of 2×YT medium supplemented with the same antibiotics, and the cells were grown to the optical density A_{600} = 0.4. Then the temperature was decreased to 30°C, and 30 min later the cells were induced by addition of 50 μM isopropyl β-D-thiogalactopyranoside. The culture was grown for 24 h, and then the cells were collected by centrifugation (10 min, 22,000*g*, 4°C).

The cell paste (2.5 g) prepared from 360 ml of the culture was resuspended in 30 ml of cell lysis buffer (100 mM Tris-HCl, pH 7.5, 1 mM EDTA). After addition of phenylmethylsulfonyl fluoride to the concentration of 1 mM, the cells were treated with lysozyme (0.5 mg/ml). Then the suspension was supplemented with NaCl to the concentration of 1 M and lysed by sonication using a UZDN-2T ultrasound generator (Ukraine). The lysate was centrifuged for 30 min under the conditions described above, the supernatant (40 ml) was supplemented with crystalline $(NH_4)_2SO_4$ to 80% saturation, kept on ice for 2 h, and centrifuged again under the same conditions. The precipitate was dissolved in buffer A (20 mM Hepes-NaOH, pH 7.5, 1 mM EDTA, 1 mM DTT) containing 1 M NaCl (final volume of the protein solution was 5 ml) and diluted with 45 ml of buffer A.

The solution was applied onto a column with 5 ml of SP-Sepharose (GE Healthcare, USA) equilibrated with buffer A containing 100 mM NaCl. The column was washed with 25 ml of the same buffer. The sorbent-bound protein was eluted with 15 ml of 800 mM NaCl in buffer A, diluted with nine volumes of buffer A, and applied onto a column with 5 ml of heparin-Sepharose (GE Healthcare) equilibrated with buffer A containing 100 mM NaCl. The protein was eluted with a 100-1000 mM NaCl gradient in buffer A. The ability of the fractions to cleave DNA containing different lesions was analyzed (see below). The most active fraction (700-800 mM NaCl) was tested for the presence of co-purifying *E. coli* AP endonucleases and for the ability to form covalent complexes with ODNs containing different modified bases (see below). Then this fraction was supplemented with nine volumes of buffer B (20 mM sodium phosphate, pH 7.4, 500 mM NaCl) and applied onto a 5-ml HiTrap chelating column charged with Ni^{2+} (GE Healthcare). The column was washed with 25 ml of the same binding buffer, and the bound protein was eluted with an imidazole gradient (50-500 mM) in the same buffer. The fractions were analyzed by electrophoresis in a 12% polyacrylamide gel, and the enzyme activity was tested. The most active fractions were collected and dialyzed against buffer containing 25 mM Tris-HCl, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 50% glycerol (v/v). The protein was stored at –20°C.

Determination of activities of Mtu-Nei2 preparations. The reaction mixture (20 μl) containing 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 2 mM EDTA, and 50 nM double-stranded or single-stranded ODN substrate was supplemented with either 2 μl of the protein fraction under analysis or Fpg or Nei to the concentration of 200 nM. The reaction was conducted for 15 min at 37°C and stopped by addition of 10 μl of formamide solution of 0.25% xylene cyanol, 0.25% bromophenol blue, and 20 mM EDTA followed by heating for 2 min at 95°C. To assay for the AP lyase activity of the protein, substrates containing the base Ura were treated with Ung (7 U/ml for 30 min at 37°C) in the reaction buffer (see above) prior to addition of *Mtu*-Nei2, and the reaction with *Mtu*-Nei2 was stopped by heating for 30 sec. To detect the possible presence of co-purifying endonuclease IV (Nfo) of *E. coli*, the protein samples were heated for 5 min at 65°C before addition to the reaction mixture. The reaction products were analyzed by denaturing PAGE with subsequent phosphorimaging of an Image Screen K screen (Kodak, USA) using a Molecular Imager FX system (Bio-Rad, USA).

Production of Mtu-Nei2 covalent complexes with damaged DNA. Each reaction mixture (20 μl) contained 50 nM double-stranded ODN analyzed, 50 mM $NaBH_4$, 25 mM potassium phosphate (pH 6.8), 1 mM EDTA, 1 mM DTT, and 2 μl of the fraction under study. To prepare a mobility marker, *OG23:C23 or *DHU23:C23 in the same buffer was supplemented with 200 nM *Eco*-Fpg or *Eco*-Nei, respectively. The reaction was conducted for 30 min at 37°C, the products were separated by 12% SDS-PAGE, and radioactivities of the bands were determined by phosphorimaging.

Complementation of *E. coli* mutator phenotypes by *mtu-nei2* and *mtu-fpg2* genes. The strains DH5α, CC104, CC104 *fpg mutY*, and SW2-38 (*nth nei*) were transformed with plasmids pET23a or pET15b, without or with the *mtu-nei2* or *mtu-fpg2* insert, respectively. The cells were grown overnight at 37°C with shaking in 5 ml of LB medium in the presence of an appropriate antibiotic. The level of mutagenesis was determined by appearance of rifampicin-resistant mutations in the *rpoB* gene as described in [22]. To determine the cell sensitivity to H_2O_2, 100 μl of the overnight culture diluted 10^5-fold was plated on a Petri dish, and a filter paper disc 7 mm in diameter was placed in the center of the dish and wetted with 10 μl of 9.8 M H_2O_2 (or with 10 μl of 9.8 M H_2O_2 and 10 μl of 1 M $FeCl_2$ or 1 M $CuCl_2$), or with 10 μl of 1 M $KMnO_4$. After overnight incubation at 37°C, a distance from the center of the disc to the nearest colonies was determined. In all cases, data for different strains were compared using Student's *t*-test.

RESULTS AND DISCUSSION

Cloning of Nei and Fpg homologs from *M. tuberculosis*. A search in the *M. tuberculosis* genome revealed four sequences homologous to the *fpg* and *nei* genes of *E. coli* (accession numbers NP_217440.1, NP_337925.1, NP_337025.1, and NP_215459.1 in the GenBank database). For a more accurate comparison of the corresponding proteins with their *E. coli* homologs, we have analyzed the conservation of the polypeptide sequence elements and motifs crucial for the specificity of Fpg and Nei as shown by biochemical and X-ray structural data [20, 23-26]. For the Fpg proteins such specific elements are represented by the N-terminal consensus sequence Pro–Glu–Leu–Pro–Glu (specific element I), the intercalating triad Met73–Arg108–Phe110 (II, here and further the numeration corresponds to positions in the *E. coli* protein), the residue Lys155 (III), and the residue Ala (or another small uncharged amino acid residue) in position 171 (IV). Specific elements for the Nei proteins include the N-terminal consensus sequence Pro–Glu–Gly (specific element I), the intercalating triad Gln69–Leu70–Tyr71 (II), the residue Ala (or another small uncharged amino acid residue) in position 155 (III), and the residue Arg171 (IV). Analysis of conservation of these elements in the aligned sequences has shown that the polypeptide NP_217440.1 belongs to the Fpg group (Fig. 1), and it is further denoted as *Mtu*-Fpg1. Crucial functional elements of the polypeptides NP_337925.1 and NP_337025.1 are combinations of the Nei and Fpg motifs. Specific elements I and III of both polypeptides from *M. tuberculosis* correspond to the proteins Nei and are homologous to the intercalating residues (element II) of both Nei and Fpg, whereas element IV corresponds to Nei in the case of polypeptide NP_337025.1 and to Fpg in the case of NP_337925.1. Because the specific elements of these two proteins are somewhat more similar to Nei than to Fpg, they are further called *Mtu*-Nei1 (NP_337925.1) and *Mtu*-Nei2 (NP_337025.1), respectively. However, similarity of the enzyme specificity to that of Nei from *E. coli* cannot be assumed. The polypeptide NP_215459.1 corresponds to the end of the N-terminal domain and to the whole C-terminal domain of the Fpg/Nei proteins (*Mtu*-Fpg2); and it retains the motifs "helix–two turns–helix" and zinc finger conserved in all members of the Fpg/Nei family and necessary for DNA binding, but lacks the catalytic N-terminal dipeptide Pro-Glu (Fig. 1). Due to the retained specific motifs II-IV, this polypeptide can be considered as a homolog of Fpg (*Mtu*-Fpg2). In the region flanking the gene *mtu-nei2* from the 5′-end, there are several stop codons in all reading frames; therefore, its coding sequence is likely full-sized and not a fragment of a longer reading frame.

A search in the GenBank database in the genomic sequences of other bacteria and a subsequent classification into the Fpg and Nei groups by conservation of the specific elements has revealed Fpg homologs in virtually all bacteria, whereas Nei occurred only in Actinobacteria and some γ-Proteobacteria. Similarly to the case of *M. tuberculosis*, genomes of various Actinobacteria contain two homologs of *fpg* and *nei*; thus, it can be concluded that several duplications have occurred in Actinobacteria with a subsequent divergence of the ancestral *fpg* gene, and then the *nei* gene has been horizontally transferred to γ-Proteobacteria and, possibly, to vertebrates, which, unlike all other eukaryotes, contain several homologs of *nei* [18, 27]. The presence of multiple *fpg* and *nei* paralogs in mycobacteria and other Actinobacteria may be due to lack of the mismatch repair system that promotes fixation of gene duplications in this group [17, 28].

PCR of the *M. tuberculosis* genomic DNA resulted in products with the expected length for all four genes under study. The sequences *mtu-nei2* and *mtu-fpg2* were cloned into plasmid vectors to continue the analysis. Their sequencing revealed a complete coincidence with the sequences of corresponding open reading frames of the H37Rv strain presented in the GenBank database, a high content of GC-pairs typical for mycobacteria, and an insignificant difference from the *E. coli* genes *fpg* and *nei* in rare codon usage (Table 1). Upon ligation of the PCR products of two other sequences (*mtu-nei1* and *mtu-fpg1*) into plasmids and transformation of *E. coli*, all resulting colonies carried vectors without these inserts, although their characteristics were virtually the same as those of *mtu-nei2* and *mtu-fpg2* (Table 1). Possibly, this suggests toxicity of the proteins *Mtu*-Nei1 and *Mtu*-Fpg1 for *E. coli* cells even at a low level of the non-induced synthesis.

Complementation of the *E. coli* mutator phenotype. Possible functions of *Mtu*-Nei2 and *Mtu*-Fpg2 were determined by their ability to complement the spontaneous mutator phenotype of *E. coli* strains deficient in different genes responsible for the repair of DNA oxidative damages: CC104 *fpg mutY* and SW2-38 (*nth nei*). The former strain is deficient in the repair of oxidized purines and the latter is deficient in the repair of oxidized pyrimidines because of inactivation of the respective DNA glycosylases. The strains were transformed by the pET series plasmids carrying the genes *mtu-nei2* or *mtu-fpg2*. These plasmids contain a low efficiency cryptic promoter of the *E. coli* RNA polymerase, which maintains synthesis of small amounts of the target mRNA even in the absence of induction of the phage T7 RNA polymerase from the promoter *lac*UV5-T7 of the prophage DE3 [29]. The repair-proficient CC104 strain gave a small number of spontaneous revertants capable of growing on a rifampicin-containing medium, whereas the isogenic strain CC104 *fpg mutY* displayed a high level of mutagenesis (Table 2) that was in agreement with the literature [30, 31]. Transformation of the strain CC104 *fpg mutY* with the plasmids pET-23a or pET-15b was not associated with significant changes in the mutation frequency, whereas a

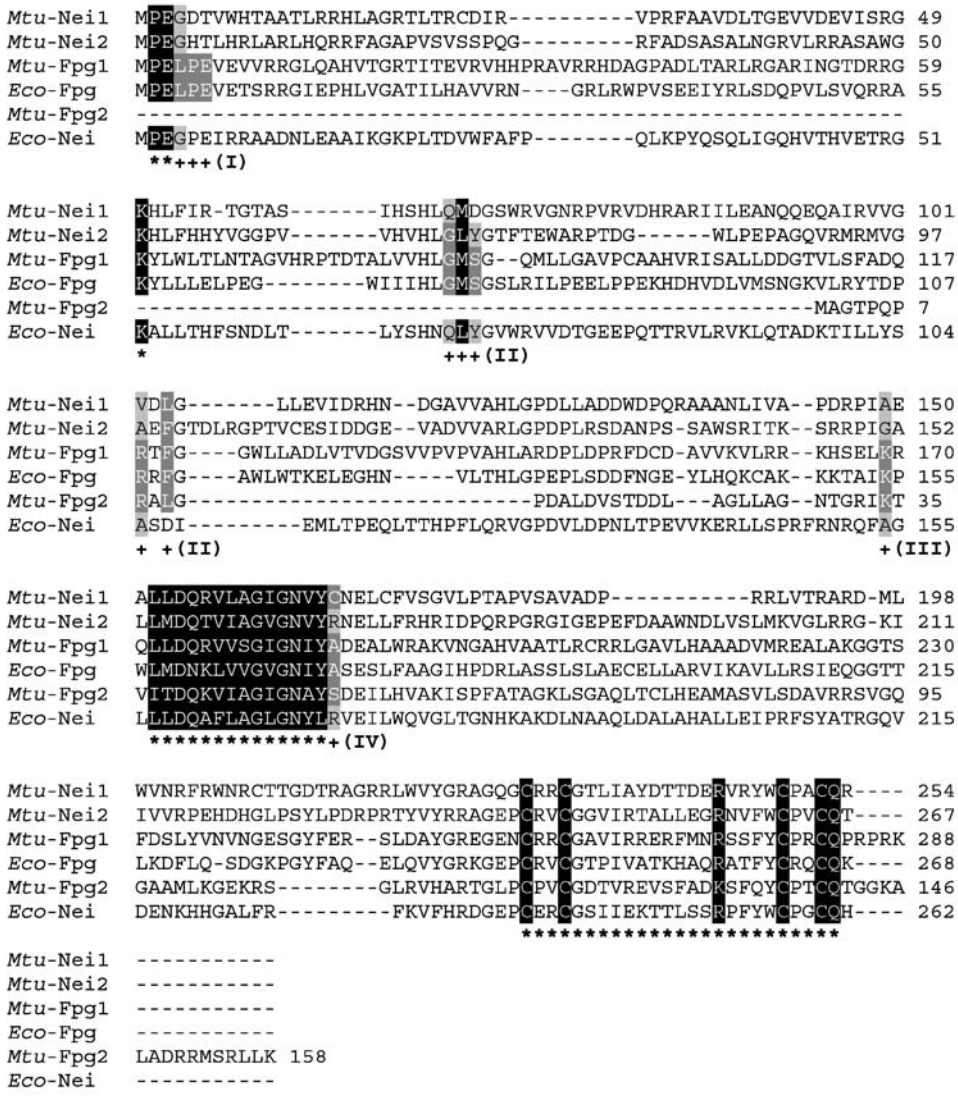

Fig. 1. Comparison of amino acid sequences of proteins of the Fpg and Nei groups in the homologs from *M. tuberculosis*. Residues highly conserved in all Fpg and Nei proteins and involved in catalysis or DNA binding are indicated by black background and asterisks. The first of the long homology blocks (Leu157–Tyr170 by the *Eco*-Fpg numeration) corresponds to the central part of the "helix–two turns–helix" motif, the second block (Cys243–Gln267 by the *Eco*-Fpg numeration) corresponds to the zinc finger motif. Symbols "+" indicate the residues of the Fpg or Nei specific elements: I is the N-terminal helical region adjacent to the catalytic dipeptide Pro-Glu, II are intercalating residues, III and IV are specific residues responsible for the correct orientation of the zinc finger. Residues specific for Fpg and for Nei are shown, respectively, by white letters on the dark-gray background and by black letters on the light-gray background. Residue numbers start from the N-terminus exposed after the initiating Met residue is cleaved off.

plasmid with the *mtu-nei2* insert decreased the mutation frequency by two orders of magnitude ($p < 0.05$). Although in *E. coli* DNA containing oxidized purine bases is not a substrate for Nei, this enzyme can excise 8-oxoG from double-stranded ODN with a low efficiency. A decrease in the level of spontaneous mutagenesis in the CC104 *fpg mutY* strain suggests that Mtu-Nei2 also could excise 8-oxoG (see below) or other oxidized purine bases (e.g. Fapy-derivatives of Gua and Ade) from DNA.

Transformation of the CC104 *fpg mutY* strain with the plasmid containing the *mtu-fpg2* insert resulted in a slight (~1.5-fold) but statistically significant increase in the number of mutant colonies (Table 2), but the transformation of the *E. coli* strain DH5α did not change the level of spontaneous mutagenesis compared to the untransformed cells. It has been mentioned that *Mtu*-Fpg2 lacks the catalytic dipeptide Pro-Glu but retains most of the structural elements required for recognition of 8-oxoG and binding with DNA. It cannot be excluded that in the cells deficient in components of the GO-system *Mtu*-Fpg2 can recognize an oxidized base, bind it, and prevent its repair through other minor pathways

(nucleotide excision repair [32] or mismatch repair [33]). On the other hand, 8-oxoG is efficiently removed from DNA of the wild type DH5α cells by the BER system, and no pro-mutagenic effect of *Mtu*-Fpg2 is observed.

The level of mutagenesis in the SW2-38 cells (*nth nei*) was one to two orders of magnitude higher than in the wild type strains CC104 and DH5α, which confirmed the literature data [34]. Differences in the mutagenesis levels in the cells transformed by plasmids with and without the inserted sequence *mtu-nei2* or *mtu-fpg2* were insignificant. Oxidized pyrimidines are, as a rule, cytotoxic and not mutagenic lesions; therefore, we have compared the ability of plasmids pET-23a, pET-23a-*mtu-nei2*, pET-15b, and pET-15b-*mtu-fpg2* to increase the survival of *E. coli* SW2-38 and CC104 *fpg mutY* under conditions of oxidative stress caused by H_2O_2 (in the presence or absence of Fe^{2+} or Cu^{2+}) or $KMnO_4$. No significant differences were detected between the strains transformed by plasmids with or without the insert (data not shown). Thus, we suggest that *Mtu*-Nei2 and *Mtu*-Fpg2 are not involved in the repair of oxidized pyrimidine bases, at least of those generated in DNA treated by H_2O_2 or $KMnO_4$ under aerobic conditions. As shown below, the best substrate for *Mtu*-Nei2 is DNA with 5,6-dihydrouracil (DHU), which is barely produced in aqueous solutions containing O_2 [35].

Table 1. Contents of GC-pairs and rare codons in the sequences of Fpg and Nei homologs from *M. tuberculosis*

Gene	Codons used in *E. coli* with the incidence, %		% GC
	<10%	10-20%	
eco-fpg	1.1	6.3	53.8
eco-nei	0.4	3.8	52.6
mtu-fpg1	0.3	6.2	67.1
mtu-fpg2	0.0	4.1	66.5
mtu-nei1	1.2	3.5	67.8
mtu-nei2	0.4	7.1	66.8

Table 2. Complementation of *E. coli* spontaneous mutator phenotype with *mtu-nei2* and *mtu-fpg2* genes

Strain (genotype)	Mutation frequency*
SW2-38 (*nth nei*)	$(1.1 \pm 0.4) \cdot 10^{-7}$
SW2-38 (*nth nei*) pET-23a	$(4.3 \pm 1.4) \cdot 10^{-7}$
SW2-38 (*nth nei*) pET-23a-*mtu-nei2*	$(3.9 \pm 1.5) \cdot 10^{-7}$
CC104	$(2.0 \pm 4.5) \cdot 10^{-8}$
CC104 *fpg mutY*	$(1.1 \pm 1.7) \cdot 10^{-5}$ **
CC104 *fpg mutY* pET-23a	$(1.8 \pm 1.0) \cdot 10^{-6}$ **
CC104 *fpg mutY* pET-23a-*mtu-nei2*	$(4.9 \pm 3.8) \cdot 10^{-7}$ **, ***
CC104 *fpg mutY* pET-15b	$(2.0 \pm 1.1) \cdot 10^{-6}$ **
CC104 *fpg mutY* pET-15b-*mtu-fpg2*	$(3.1 \pm 0.6) \cdot 10^{-6}$ **, ***
DH5α	$(4.6 \pm 7.8) \cdot 10^{-8}$
DH5α pET-15b	$(2.6 \pm 1.5) \cdot 10^{-8}$
DH5α pET-15b-*mtu-fpg2*	$(1.0 \pm 1.4) \cdot 10^{-8}$

* Mean ± standard deviation ($n = 5$).

** Significant difference ($p < 0.05$) compared to the frequency of appearance of rifampicin-resistant colonies in the isogenic wild type strain.

*** Significant difference ($p < 0.05$) compared to the frequency of appearance of rifampicin-resistant colonies in the strain transformed by the same plasmid lacking the insert.

Isolation and characteristics of the recombinant *Mtu*-Nei2 protein. Because the sequence of *Mtu*-Fpg2 contains no crucial catalytic N-terminal dipeptide Pro-Glu, possible substrate specificity was studied only for *Mtu*-Nei2. The protein was expressed in *E. coli* and isolated by successive chromatography on SP-Sepharose and heparin-Sepharose. Both *Eco*-Fpg and *Eco*-Nei can excise a damaged base (DNA glycosylase activity) and cleave the sugar–phosphate backbone of DNA at the resulting apurine-apyrimidine (AP) site (AP lyase activity). During the reaction, these two enzymes form a Schiff base with the C1′ atom of the damaged deoxynucleotide, and this intermediate can be reduced by $NaBH_4$ with production of a stable covalent complex between the protein and the substrate. *Mtu*-Nei2 is expected to display a similar activity. The ability of protein fractions to cleave double-stranded ODN containing the 8-oxoG:C and DHU:G pairs which are efficiently cleaved by *Eco*-Fpg and *Eco*-Nei was used as an activity assay during purification [26, 36].

Electrophoresis in the Laemmli system showed that the most active fraction eluted during the chromatography on heparin-Sepharose at ~700-800 mM NaCl was not homogenous. To exclude a possibility of the substrate cleavage by some co-purifying DNA glycosylases of *E. coli*, the formation of covalent complexes with double-stranded ODN substrates containing different modified bases was studied (Fig. 2). On reduction by $NaBH_4$, *Mtu*-Nei2 formed stable covalent complexes with double-stranded ODNs containing both the 8-oxoG:C and DHU:G pairs, but the reaction with the latter substrate was more efficient. The mobility of these covalent complexes was lower than the mobility of complexes formed by the enzymes *Eco*-Fpg and *Eco*-Nei with their respective substrates (Fig. 2). The appearance of a highly mobile minor band in the reaction with DHU:G can be due to endonuclease III, which is the only *E. coli* enzyme besides Nei capable of excising DHU from DNA (molecular weight 23.6 kD). Molecular weight of *Mtu*-Nei2 cal-

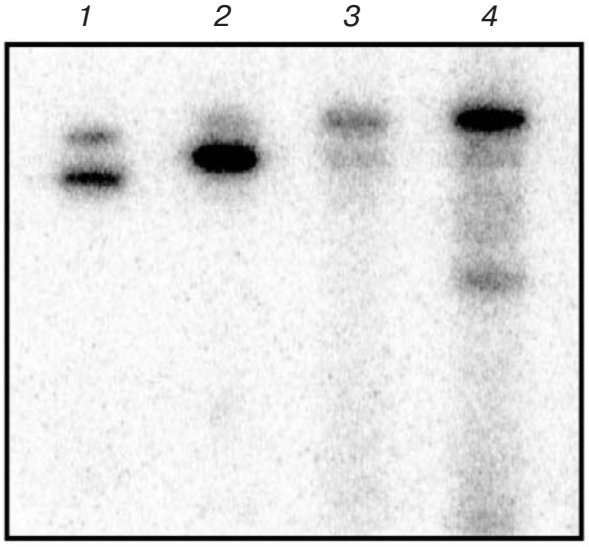

Fig. 2. Interactions of the enzymes *Eco*-Fpg, *Eco*-Nei, and *Mtu*-Nei2 with substrates resulting in formation of covalent complexes. SDS-PAGE of reaction mixtures: *1*) *Eco*-Fpg + double-stranded ODN with 8-oxoG:C; *2*) *Eco*-Nei + double-stranded ODN with DHU:G; *3*) *Mtu*-Nei2 + double-stranded ODN with 8-oxoG:C; *4*) *Mtu*-Nei2 + double-stranded ODN with DHU:G. Under the reaction conditions, *Eco*-Fpg produces two conjugates corresponding to the enzyme cross-linked to a full-size ODN (upper band) and with ODN after β-elimination (lower band), whereas *Eco*-Nei forms only one conjugate with a full-size ODN. Molecular weights of *Eco*-Fpg and *Eco*-Nei are 30.2 and 29.7 kD, respectively. The migration distance of the free double-stranded ODN is longer than the gel length.

culated from the sequence is 29.7 kD, which is close to the molecular weight of *Eco*-Nei and slightly less than the molecular weight of *Eco*-Fpg (30.2 kD). We have suggested that during the *mtu-nei2* mRNA translation, the termination on its own stop codon is inefficient and the resulting longer polypeptide should be terminated on the plasmid pET-23a stop codon. This should be associated with addition of 20 amino acid residues to the C-terminus of the protein, including the His_6 peptide encoded in the polylinker sequence of the plasmid. Therefore, an additional chromatography was performed on a Ni^{2+}-containing chelating sorbent. The most active fraction eluted from the column with ~200 mM imidazole was used for the further work.

To compare the substrate specificity of *Mtu*-Nei2 to those of the other enzymes of the Fpg/Nei family, we have studied the ability of the *Mtu*-Nei2 preparation to cleave double-stranded and single-stranded ODN substrates containing different lesions and different bases opposite them (Fig. 3). Similarly to the homologous enzymes from *E. coli*, *Mtu*-Nei2 displayed a high AP lyase activity on AP-containing substrates. The activity of the preparation on the substrates with AP-sites was unaffected by EDTA, but disappeared after heating to 65°C, indicating the absence of contamination by two known AP endonucleases of *E. coli*, the Mg^{2+}-dependent enzyme Xth and the thermostable enzyme Nfo. The preparation under study efficiently excised DHU residues from double-stranded ODNs, using DHU:G as a preferred substrate, which is similar to the *Eco*-Nei specificity for the base opposite to DHU [26]. The enzyme was also capable of cleaving double-stranded ODN containing 8-oxoG and U, but less efficiently. The *Mtu*-Nei2 preparation was inactive on double-stranded ODNs containing 8-oxoA lesions and did not excise adenine from pairs with DHU, 8-oxoG, and 8-oxoA; i.e. it had no activity similar to that of MutY DNA glycosylase [37]. The preparation also cleaved single-stranded ODNs containing AP and U, but less efficiently than double-stranded ODNs with these lesions; a slight activity of the enzyme was also detected on DHU, 8-oxoG, and 8-oxoA within single-stranded ODNs (Fig. 3).

The substrate specificities of *Eco*-Fpg and *Eco*-Nei are now well characterized. Oxidized purine bases are the main substrates for the former enzyme and oxidized pyrimidine bases are substrates for the latter. However, both *Eco*-Fpg and *Eco*-Nei can excise oxidized bases belonging to the substrates of the other but with lower efficiency: for example, *Eco*-Fpg excises DHU bases from double-stranded ODNs [38, 39] and *Eco*-Nei excises 8-oxoG bases [26, 40]. Neither enzyme is active toward 8-oxoA, U, and A within non-canonical pairs. It can be concluded that the substrate specificity of *Mtu*-Nei2 better resembles that of *Eco*-Nei than of *Eco*-Fpg, because it efficiently excises DHU bases and is much less active on 8-oxoG. On the other hand, the ability of *Mtu*-Nei2 to hydrolyze single-stranded substrates makes it different from *Eco*-Nei and *Eco*-Fpg, which very inefficiently cleave damaged single-stranded ODNs [41]. In addition, neither *Eco*-Fpg, nor *Eco*-Nei can remove Ura bases from DNA.

Induction of oxidative burst in macrophages results in production of large amounts of reactive oxygen and nitrogen species and hypohaloids [9]. Since DNA repair in infecting bacteria is crucial for successful invasion, it was supposed that DNA should be one of the main targets of these oxidizers [9]. However, it is virtually unknown which lesions arise in DNA during the oxidative burst. Activated macrophages are directly shown to induce generation of 8-oxoG, 5,6-saturated pyrimidines and single-stranded breaks in DNA [42, 43], but the spectrum of lesions is likely wider. Homologs of Fpg and Nei from *M. tuberculosis* may be involved in the repair not only of 8-oxoG and DHU but also of other lesions not investigated in the present work, which appear under conditions of oxidative stress.

The importance of BER for infectivity of bacteria is underscored by the fact that Ung deficiency leads to a decrease in survival of *Pseudomonas aeruginosa* and *Mycobacterium smegmatis* in macrophages due to increased sensitivity of the infectious agent to reactive

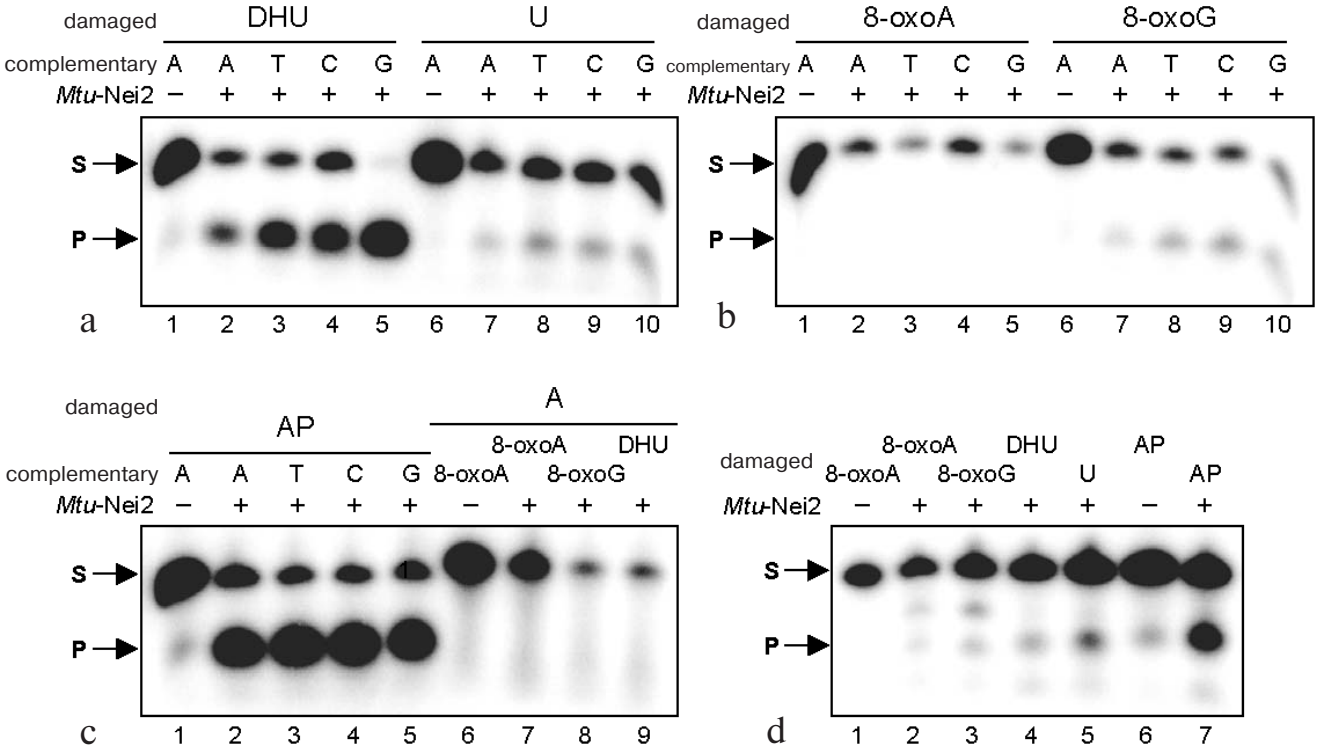

Fig. 3. Substrate specificity of *Mtu*-Nei2. The cleavage of different double-stranded substrates containing: a) DHU (lanes *1-5*) and U (*6-10*) opposite to A (lanes *1*, *2*, *6*, *7*), T (*3*, *8*), C (*4*, *9*), and G (*5*, *10*); b) 8-oxoA (lanes *1-5*) and 8-oxoG (*6-10*) opposite to A (lanes *1*, *2*, *6*, *7*), T (*3*, *8*), C (*4*, *9*), and G (*5*, *10*); c) AP-site (lanes *1-5*) and A (*6-9*) opposite to A (lanes *1*, *2*), T (*3*), C (*4*), G (*5*), 8-oxoA (*6*, *7*), 8-oxoG (*8*), DHU (*9*); and d) single-stranded ODNs containing 8-oxoA (lanes *1*, *2*), 8-oxoG (*3*), DHU (*4*), U (*5*), and AP-site (*6*, *7*). The reaction conditions are described in "Materials and Methods".

nitrogen species [14]. *Salmonella typhimurium* also can persist in macrophages, and the bacteria deficient in the genes *nfo* and *xth* coding for AP endonucleases (enzymes responsible for the BER step next to base excision) display the lower infectivity in mice [44]. Thus, the BER system assists the survival of bacteria under conditions of oxidative stress and can be a promising target for development of novel drugs.

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