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NOTES

Characterization of Endonuclease III (*nth*) and Endonuclease VIII (*nei*) Mutants of *Escherichia coli* K-12

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The *nth* and *nei* genes of *Escherichia coli* affect the production of endonuclease III and endonuclease VIII, respectively, glycosylases/apurinic lyases that attack DNA damaged by oxidizing agents. Here, we provide evidence that oxidative lethal lesions are repaired by both endonuclease III and endonuclease VIII and that spontaneous mutagenic lesions are repaired mainly by endonuclease III.

Endonuclease III (endo III) has an apurinic (AP) lyase activity at apurinic/apyrimidinic sites in DNA and has a DNA glycosylase activity that excises oxidative pyrimidine residues such as thymine glycol (2, 5, 8, 11, 14). *Escherichia coli* mutants defective in endo III (*nth* mutants) were found to exhibit normal resistance to DNA-damaging agents such as hydrogen peroxide and X rays (7). Since thymine glycol has been shown to be a lethal lesion in vitro (6, 12, 13) and in vivo (10, 15, 17), and a mutagenic lesion in vitro (4), the possibility existed that alternative pathways for the repair of these damages are present in *E. coli*. The availability of *nth* mutants facilitated the identification of a distinct enzyme with similar properties, designated endonuclease VIII (endo VIII) (16). Endo VIII releases modified pyrimidine bases and possesses DNA glycosylase and AP lyase activities. The gene coding for endo VIII was named *nei.*

To understand the role of thymine glycol and modified pyrimidine bases in lethality and mutagenesis, it is important to determine the properties of *nei* and *nth* single mutation and *nei nth* double mutation. We report here the cloning and sequencing of the *nei*$^+$ gene, coding for endo VIII, and the use of the cloned gene to construct an endo VIII-defective mutant of *E. coli.*

Isolation of *nei* and *nth* plasmids. The structural gene for endo III (*nth*) of *E. coli* was assigned on Clark-Carbon plasmid pLC3-6, which contains DNA fragments at 36 min on the linkage map (18). Therefore, using pLC3-6, a 5-kb *Eco*RI-*Kpn*I fragment including the *nth* gene was ligated on pTZ18R, named pTZ18R*nth*. The sequence of a portion of this fragment agreed perfectly with that of the *nth* gene (data not shown).

The DNA sequence of the structural gene coding for endo VIII (*nei*) of *E. coli* appears in the GenBank database under accession no. U38616. We have previously cloned a 20-kb DNA fragment, named pKY1, containing the structural gene coding for photolyase (*phr*) of *E. coli*, which is mapped at 16 min (22). Part of the detailed physical map of pKY1 looks like that of *nei*. We therefore subcloned the 5-kb *Pst*I fragment from pKY1 to pTZ18R. The DNA sequence of the fragments completely agreed with that of U38616. The detailed restriction map is shown in Fig. 1. Thus, plasmid pTZ18R*nei* carries the *nei*$^+$ gene. This finding further shows that the location of the *nei* gene is about 5 kb clockwise from the *phr* gene.

Construction of *nei* and *nth* mutants. In the 5-kb *Eco*RI-*Kpn*I fragment containing *nth*$^+$, there are two *Pst*I sites, one within the *nth* gene and the other 37 bp upstream from the ATG start codon. The *nth*$^+$ gene was inactivated by cloning a 1.5-kb chloramphenicol resistance (Cm^r) cassette from pKY1291 into two *Pst*I sites. pTZ18R*nth*::Cm^r, the resulting insertion mutant, was used to replace a genomic *nth*$^+$ allele in JC7623 (*recBC sbcB*) by gene replacement (1, 21). AB1157 was then P1 transduced with the *nth*::Cm^r insertion mutant via selection for Cm^r.

To construct an *nei* mutant, we used plasmid pBG6, which lacks *nei* (Fig. 1). To facilitate the selection of the *nei* mutant, a kanamycin resistance (Km^r) cassette was inserted into the pBG6 *Bgl*II site to make pBG6::Km^r. pBG6::Km^r was used to replace a genomic *nei*$^+$ allele, and strains with the Δ*nei*::Km^r allele were constructed by P1 phage transduction.

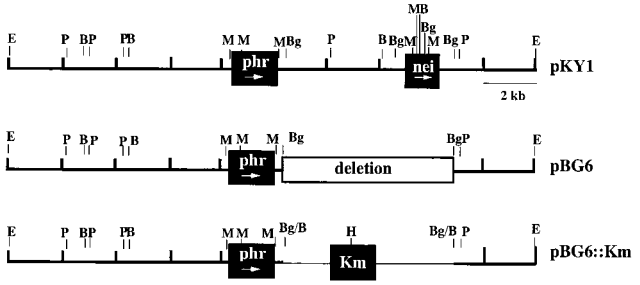

FIG. 1. Restriction map of the *phr* and *nei* regions in plasmid pKY1 (22). Restriction sites and coding regions for *phr* and *nei* shown as solid boxes were deduced from the previous mapping and present sequence data. Plasmid pBG6 was obtained by deleting an about 5-kb *Bgl*II DNA fragment (open box) including *nei*. Plasmid pBG6::Km^r resulted from the cloning of a 1.5-kb *Bam*HI/*Bam*HI fragment containing a Km^r marker (solid box with thin line) from pKY1292 in the unique *Bgl*II site of pBG6. Abbreviations for restriction enzyme sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; M, *Mlu*I; P, *Pst*I; Bg/B, ligation between *Bgl*II and *Bam*HI. The arrows show transcription orientation.

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TABLE 1. Thymine glycol glycosylase/AP lyase activities in crude lysates of mutants

Strain	Relevant genotype	Relative activity[a]
AB1157	Wild type	100
NKJ1002	*nth*::Cm^r	13
NKJ1003	Δ*nei*::Km^r	65
NKJ1004	*nth*::Cm^r Δ*nei*::Km^r	0.3
NKJ1004(pACYC*nth*)[b]	*nth*::Cm^r Δ*nei*::Km^r *nth*$^+$	620
NKJ1004(pACYC*nei*)[b]	*nth*::Cm^r Δ*nei*::Km^r *nei*$^+$	215

[a] Percentage of the specific enzyme activity of a strain relative to that of AB1157. The activity of AB1157 was 123 nicks/min/mg of protein.
[b] Endo III or endo VIII was amplified by introducing plasmids into the *nth nei* double mutant.

Thymine glycol endonuclease activity in the *nei* and *nth* mutants. Thymine glycol glycosylase/AP lyase activities of these mutants were assayed, and the results are shown in Table 1. Most of the enzyme activity in the wild-type cell was due to expression of endo III, and 10 to 40% was due to expression of endo VIII. The result is consistent with a previous observation (7) that the *nth* mutant had about 20 to 34% of the parental level of activity. The *nth nei E. coli* cell extracts showed less than 1% of the wild-type level of activity. Since *E. coli* strains with Cm^r and Km^r alleles can decrease and the *nth* plasmid and *nei* plasmid can overproduce thymine glycol glycosylase/AP lyase, are concluded that the *nth* and *nei* genes on the *E. coli* chromosome were disrupted.

Sensitivity to oxidative damaging agents. It was shown previously that the *nth* mutants were about as resistant as wild-type cells to ionizing radiation and to H_2O_2 (7). Consistent with these results, NKJ1002 (*nth*::Cm^r) and NKJ1003 (Δ*nei*::Km^r) were as resistant as AB1157 to 10 mM H_2O_2. NKJ1004 (*nth*::Cm^r Δ*nei*::Km^r) was extremely sensitive to H_2O_2 (Fig. 2A). As a control, exonuclease III (*xth*) mutant BW9109 was as sensitive as NKJ 1004 to H_2O_2. Similar findings that NKJ1002 and NKJ1003 were as resistant as AB1157 and that NKJ1004 was extremely sensitive were obtained after irradiation with X rays

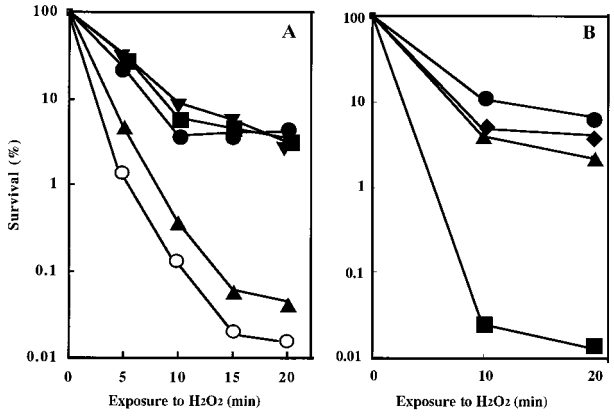

FIG. 2. Sensitivity to H_2O_2 of the *nei* and *nth* single-mutant and *nei nth* double-mutant strains. The cells were grown to logarithmic phase and treated with 10 mM H_2O_2 at 37°C without shaking. Experiments were carried out three to five times, and the results were reproducible. The results represent one representative experiment. (A) AB1157 (wild-type; ●), NKJ1002 (*nth*::Cm^r; ■), NKJ1003 (Δ*nei*::Km^r; ▼), NKJ1004 (*nth*::Cm^r Δ*nei*::Km^r; ▲), and BW9109 (Δ*xth*; ○). (B) AB1157(pACYC177) (wild-type with pACYC177; ●), NKJ1004 (pACYC177) (*nth*::Cm^r Δ*nei*::Km^r with pACYC177; ■), NKJ1004(pACYC*nth*) (*nth*::Cm^r Δ*nei*::Km^r with pACYC*nth*; ▲), and NKJ1004(pACYC*nei*) (*nth*::Cm^r Δ*nei*::Km^r with pACYC*nei*; ◆).

TABLE 2. Spontaneous mutagenesis in *nei* and *nth* mutants

Strain	Relevant genotype	Arg$^+$ His$^+$ revertants/10^8 cells[a]
AB1157	Wild type	3.2
NKJ1002	*nth*::Cm^r	24.8
NKJ1003	Δ*nei*::Km^r	1.7
NKJ1004	*nth*::Cm^r Δ*nei*::Km^r	26.8
AB1157(pACYC177)[b]	Wild type	1.5
NKJ1002(pACYC177)[b]	*nth*::Cm^r	55.7
NKJ1002(pACYC*nth*)[b]	*nth*::Cm^r *nth*$^+$	4.7
NKJ1002(pACYC*nei*)[b]	*nth*::Cm^r *nei*$^+$	33.1
NKJ1004(pACYC177)[b]	*nth*::Cm^r Δ*nei*::Km^r	16.4
NKJ1004(pACYC*nth*)[b]	*nth*::Cm^r Δ*nei*::Km^r *nth*$^+$	2.8
NKJ1004(pACYC*nei*)[b]	*nth*::Cm^r Δ*nei*::Km^r *nei*$^+$	7.9

[a] Average of three independent experiments.
[b] Endo III or endo VIII was amplified by introducing an *nth* or *nei* plasmid.

(data not shown). Plasmids carrying either the *nth* or the *nei* gene can complement the extreme sensitivity of NKJ1004 to H_2O_2 (Fig. 2B). Thus, the results strongly indicate the biological significance of the *nei* gene as well as the *nth* gene in oxygen species DNA repair.

Spontaneous mutagenesis. An *nth* mutant is known to have a weak mutator effect (7). This mutator effect was not enhanced by introduction of the *nei* mutation (Table 2). The *nei* mutant is not a mutator at least in the allele tested. The mutator effects of NKJ1002 and NKJ1004 were suppressed by introduction of the *nth* plasmid. The *nei* plasmid partly suppressed the mutator effect of these strains when *argE3 his-4*-to-Arg$^+$ His$^+$ (Table 2), rifampin-sensitive to rifampin-resistant (data not shown), and nalidixic acid-sensitive to nalidixic acid-resistant (data not shown) mutations were measured. The mutations from *argE his-4* to Arg$^+$ His$^+$ have been shown to arise from GC-to-AT transition (20). Cytosine or guanine oxidative adducts may be the spontaneous mutagenic lesions involved. The results further suggest that the mutagenic lesions can be repaired by endo III and partly or weakly by endo VIII.

Since we inserted the Km^r gene at 5-kb *Bgl*II sites including *nei*, the fragment may include *nei* along with other genes which control for repair of oxidized pyrimidines. The possibility that all of the phenotypes seen in the *nei* mutation may be ascribable to the loss of such unknown genes but not the loss of endo VIII exists.

Schizosaccharomyces pombe, *Saccharomyces cerevisiae*, and human homologs of *E. coli* endo III were recently cloned and characterized (3, 9, 19). These results suggest that there is a homology family of endo III-like DNA repair enzymes present throughout phylogeny. With regard to endo VIII, a computer search using the BLAST program did not reveal any eucaryotic proteins with similarity to endo VIII. Thus, it is not known whether both endo III and endo VIII or only endo III can operate to protect against oxygen attacks in eucaryotic cells.

Nucleotide sequence accession number. The DNA sequence of the *nei* region examined in this study has been submitted to DDBJ/EMBL/GenBank under accession no. D89754.

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