Volume 17 Number 15 1989 Nucleic Acids Research

Cloning and characterization of *mutL* and *mutS* genes of *Vibrio cholerae*: nucleotide sequence of the *mutL* gene

Tapan Kumar Bera, Subrata K.Ghosh+ and Jyotirmoy Das*

Biophysics Division, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Calcutta 700032, India

Received May 24, 1989; Revised and Accepted July 14, 1989 EMBL accession no. X15438

ABSTRACT

The mutL and mutS genes of Vibrio cholerae have been identified using inter-specific complementation of Escherichia coli mutL and mutS mutants with plasmids containing the gene bank of V. cholerae. The recombinant plasmid pJT470, containing a 4.7 kb fragment of V. cholerae DNA codes for a protein of molecular weight 92,000. The product of this gene reduces the spontaneous mutation frequency of the E. coli mutS mutant. The plasmid, designated pJT250, containing a 2.5 kb DNA fragment of V. cholerae and coding for a protein of molecular weight 62,000, complements the mutL gene function of E. coli mutL mutants. These gene products are involved in the repair of mismatches in DNA. The complete nucleotide sequence of mutL gene of V. cholerae has been determined.

INTRODUCTION

Noncomplementary base pairing in DNA occurs either due to replication error or during recombination between homologous but non-identical DNA sequences or due to chemical modification of bases, such as, deamination of 5'-methyl-cytosine to thymine. These mismatches, if not repaired, result in high spontaneous mutation frequency. The repair has to be on the newly synthesized strand of the DNA molecule, otherwise the error will be fixed permanently. A number of mechanisms have been proposed for the repair of such DNA mismatches and functions of several gene products are involved in these repair processes (1-3).

DNA strand discrimination is done in different organisms in different ways. While in Escherichia coli, transient under methylation of the adenine residue in the sequence GATC by the product of the DNA adenine methylase gene (dam) helps to recognize the daughter strand (4-7), in Streptococcus pneumonia, single strand breaks direct repair to donor strand in transformation and to the newly synthesized strand in replication (3,8). One of the mechanisms for repairing mismatches in DNA, the methyl directed repair process, requires the function of mutS, mutH, mutL and mutU (uvrD) genes besides the dam

TABLE 1
Bacterial strains and plasmids used

Strain	Genotype	Source or reference
E. coli K12		
GW3732	F^- thr-1 leu6 proA2 his4 thi1 argE3 lacY1 galK2 ara14 xyl-5 mtl-1 tsx-33 rpsL31 supE44 mutS201::Tn5	23
GW3734	F^- thr-1 leu6 proA2 his4 thi1 argE3 lacY1 galK2 ara14 xyl-5 mtl-1 tsx-33 rpsL31 supE44 mutL211::Tn5	23
JM101	supE thi Δ (lac proA,B)/F' traD36 proA,B $lacI^q$ Z M15	24
CSR603	thr-1 ara14 leuB6Δ (gpt-proA)62 lacY1 tsx33 supE44 phr-1 galK2 λ^- rac^- gyrA98 recA1 rpsL31 kdgK51 xyl-5 mtl-1 uvrA6	25
GM33	dam3 argE3 thi1	
C600	thr leu thi lacY	
*V. cholerae**		
569B	Serotype, Inaba, Prototroph, hypertoxinogenic	
154	Serotype, Ogawa, Prototroph, mildly toxinogenic	
IKWKI	Serotype, Inaba	
H218	Serotype, Inaba	
NIH41	Serotype, Ogawa, lysogenic for phages VCA1 and VCA2	
NIH35A3	Serotype, rough	
NIH35A35A	Serotype, Inaba	
*V. eltor**		
MAK757	Serotype, Ogawa, mildly toxinogenic	
EW6	Serotype, Ogawa, non-toxinogenic	
Plasmids		
pGW1811	pBR322 derivative but contains mutS gene of *Salmonella typhimurium* - Amp^r	23
pGW1842	pBR322 derivative containing mutL gene of *S. typhimurium* - Amp^r	23
pUC8	... Amp^r	24
pJT470	pUC8 derivative containing mutS gene of *Vibrio cholerae* - Amp^r	Present study
pJT250	pUC8 derivative but contains mutL gene of *V. cholerae* - Amp^r	Present study
pJT4701	pUC8 derivative but contains mutated mutS gene of *V. cholerae* - Amp^r	Present study
pJT2501	pUC8 derivative but contains mutated mutL gene of *V. cholerae* - Amp^r	Present study

*All *V. cholerae* and *V. eltor* strains were obtained from the Cholera Research Centre, Calcutta, India.

gene function (9-11). These gene products act on unmethylated or hemimethylated DNA and not on fully methylated DNA (1,2). While some in vitro studies have suggested that *mutS* and may be *mutL* gene products are involved in recognizing the mismatch in the DNA (12), their role in methyl directed DNA

mismatch repair is still not clear. The mutH gene product introduces a nick at the 5' end of the GATC sequence proximal to the mismatch (13). The mutU gene product is DNA helicase II (14,15). MutL and mutS gene products are also involved in very short patch repair (16,17).

Vibrio cholerae, a highly pathogenic gram negative bacterium and the causative agent of cholera, is inefficient in repairing UV-induced DNA damage (18-22). The spontaneous mutation frequency for any given marker, however, is comparable to that of other organisms. In view of the fact that the spontaneous mutation frequency is directly related to the ability of a cell to repair mismatches in its DNA, the status of DNA mismatch repair mechanism in this organism has been examined. These cells possess both the dam and the DNA cytosine methylase (dcm) gene products (unpublished observation). The present report describes the cloning and characterization of mutS and mutL genes of V. cholerae.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. The V. cholerae cells were grown and maintained as described previously (26). E. coli cells were grown either in Luria broth (LB) or in YT medium. Cell growth was assayed as colony forming units (CFU) in LB-agar plates.

Hydroxylamine mutagenesis

Plasmids pJT470 and pJT250 were mutagenized in vitro by hydroxylamine (27). Plasmid DNA was dissolved in 1M sodium acetate buffer (pH 4.9) to give a final concentration of 10 ug per 50 ul. To 50 ul of the plasmid solution, 150 ul of 2M hydroxylamine in sodium acetate buffer was added and the mixture was incubated at 55° for 1 hr. Then, the pH of the suspension was adjusted to 7.6 by adding 1M Tris-HCl, 20 mM EDTA (pH 8.0). The plasmid DNA was precipitated, washed, dissolved in TE buffer (10 mM Tris-1 mM EDTA, pH 7.6) and used for transformation. Ampicillin (80 ug/ml) was used for selection of resistant clones.

Nuclease digestion and mapping

Digestion of DNA with different endonucleases was carried out according to the instruction of manufacturers. Whenever required DNA fragments were recovered from low melting agarose gels. For construction of physical maps linkages between the restriction fragments were determined either from the analysis of partial digestion products or from restriction fragments produced by a mixture of two enzymes. Digestion of restriction fragments with Bal31 was used to confirm the linkages whenever necessary.

Nick translation

About 1 ug of desired DNA fragments were nick translated using [α-^{32}P]dATP (Amersham, U.K.) and DNA polymerase I (28). The reaction was carried out at 16° for 1 hr and the nick translated DNA was separated from unincorporated [α-^{32}P]dATP by passing through a Sephadex G-50 column.

Southern blotting

About 1-2 ug of the DNA to be analyzed was digested with the desired restriction enzyme and electrophoresed on horizontal agarose (0.7%) slab gels (30cm x 13cm x 0.5cm) at 3 V/cm. The gels were stained with ethidium bromide, irradiated with UV light to nick the DNA, denatured and blotted to nitrocellulose (29). Hybridization was carried out at 60° without formamide (28). The filters were sequentially washed with 3xSSC buffer (SSC buffer contained 0.15M NaCl and 0.015M Na-citrate) at room temperature and 2xSSC containing 0.5% SDS at 60°. The filters were dried and exposed to Kodak XR-5 film using an intensifying screen.

Dot-blot hybridization

V. cholerae and *E. coli* strains grown in appropriate agar media as single colonies onto nitrocellulose filters were lysed and hybridized with nick translated probes. Prior to hybridization the filter papers were washed in 50 mM Tris-HCl (pH 8.0) buffer containing 1M NaCl, 1 mM EDTA (pH 8.0) and 0.1% SDS. The condition of hybridization was the same as used for Southern hybridization.

Protein labelling in maxicells

Plasmid-coded proteins were examined using the maxicell system (25). Strain CSR603 carrying either the plasmid pJT470, or pJT250 or pUC8 were grown ($2x10^8$ CFU/ml) in minimal media containing 1% casamino acid. The cells were irradiated with UV light (50 J/m^2) and incubated at 37° for 1 hr. At the end of this period 200 ug/ml D-cycloserine was added and the incubation was continued for 16 hrs at 37°. The cells were then harvested, suspended in fresh sulphur depleted medium, incubated at 37° for 1 hr, labeled with 5 uCi ^{35}S-methionine/ml for another 1 hr. The labeled cells were harvested, washed, suspended in electrophoresis sample buffer and analyzed by SDS-polyacrylamide (10%) gel electrophoresis followed by autoradiography of dried gels as described previously (30).

DNA sequencing

The DNA sequence was determined by the dideoxynucleotide chain termination method (31). The 1.06 kilobase (kb) EcoRI-HindIII fragment, 0.7 kb HindIII-KpnI fragment and 0.8 kb KpnI-EcoRI fragment of the *mutL* gene were cloned into M13mp18 and M13mp19 RF DNA. The 1.06 kb EcoRI-HindIII fragment

TABLE 2
Spontaneous mutation rate of E. coli mutL and mutS mutants and that of cells carrying the mutL and mutS genes of V. cholerae

Strain	Rifampicin resistant colonies/10^8 CFU*
GW3732	408
GW3732::pJT470	23
GW3732::pJT4701	380
GW3734	280
GW3734::pJT250	18
GW3734::pJT2501	270

*Number of resistant colonies were determined by assaying CFU in plates containing 80 ug rifampicin/ml. The numbers represent an average of three independent experiments.

and 1.5 kb HindIII-EcoRI fragment were also digested separately with Sau3A1 and the resulting fragments were shotgun cloned into M13mp19.

RESULTS AND DISCUSSION

mutS and mutL genes of V. cholerae

Genomic libraries of V. cholerae DNA were constructed by cloning either EcoRI or PstI restriction fragments into the plasmid pUC8 as described previously (21). These libraries were maintained in E. coli strain JM101 and used to search for the mut genes of V. cholerae.

The mutator genes of E. coli and S. typhimurium cells examined so far can functionally complement each other. Assuming that these genes, if present in V. cholerae, would be able to complement to E. coli mutants for mut functions, interspecific complementation of E. coli mutL and mutS mutants with the plasmid containing the gene bank of V. cholerae was used to identify the mutL and mutS genes by assaying the spontaneous mutation frequency of the transformed cells.

E. coli strains GW3732 (mutS) and GW3734 (mutL) exhibit high level of spontaneous mutation frequency (Table 2). These cells were transformed with plasmids carrying V. cholerae genomic DNA fragment and transformants having reduced spontaneous mutation frequency relative to the control cells were selected for further study. The spontaneous mutation frequency was determined by scoring rifampicin and in some experiments nalidixic acid resistant cells. Several transformants of both GW3732 and GW3734 were isolated which had reduced level of spontaneous mutation frequency. A 2.5 kb EcoRI DNA fragment

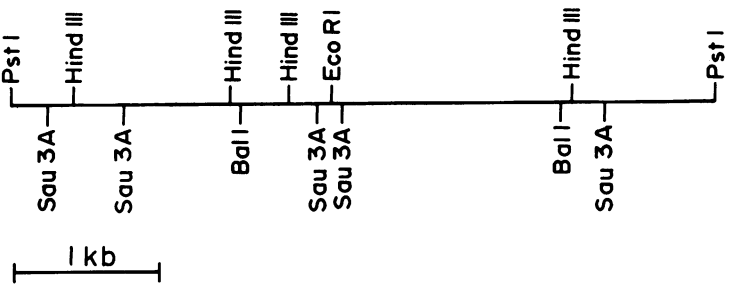

Figure 1. Restriction map of the 4.7 kb V. cholerae DNA fragment carrying the mutS gene.

of V. cholerae complemented E. coli mutL functions (Table 2). When the plasmid carrying this insert, designated as pJT250, was used to transform GW3734 cells, spontaneous mutation frequency of all transformants were reduced. A 4.7 kb PstI fragment of V. cholerae DNA complemented E. coli mutS functions. The plasmid carrying this fragment has been designated as pJT470 and GW3732 cells carrying this plasmid showed a reduction in spontaneous mutation frequency (Table 2).

To confirm that the 4.7 kb fragment of the plasmid pJT470 and the 2.5 kb fragment of the plasmid pJT250 of V. cholerae DNA indeed represent the mutS and the mutL genes respectively, these plasmids were mutagenized in vitro and used for transformation of E. coli mutL and mutS mutants. Transformants were selected as ampicillin resistant colonies. Three clones carrying the mutL genes of V. cholerae and one clone carrying the mutS gene of V. cholerae were isolated which failed to reduce the spontaneous mutation frequency of E. coli mut mutants (Table 2). The plasmids isolated from these transformants had the respective DNA fragments with almost unaltered size. These plasmids carrying the mutated mutS and mutL genes of V. cholerae are designated as pJT4701 and pJT2501 respectively.

To investigate whether all biotypes and serotypes of V. cholerae possess the mutL and mutS gene functions, the 4.7 kb mutS and 2.5 kb mutL DNA fragments were nick translated and used as probes for dot blot hybridization. All strains of V. cholerae examined so far hybridized with mutS and mutL DNA sequences of the hypertoxinogenic strain 569B of classical vibrios. Interestingly, although the mutS and mutL gene products of V. cholerae functionally complemented E. coli mutants, they poorly hybridized with E. coli genes suggesting nucleotide sequence divergence.

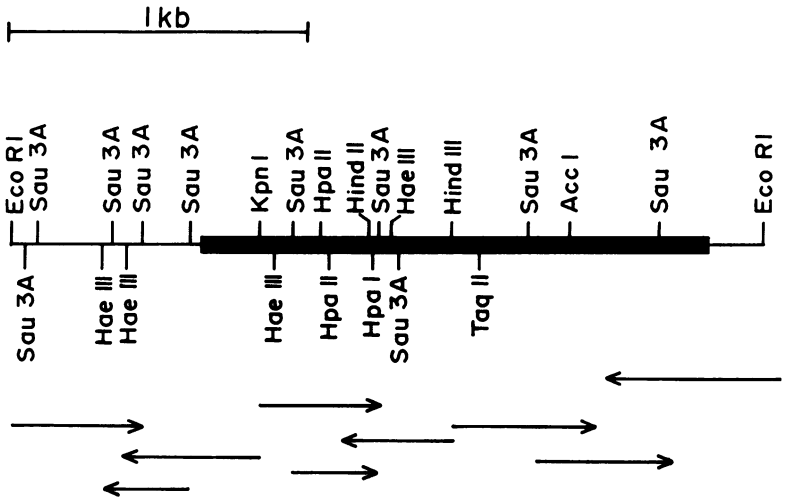

Figure 2. Restriction map of the 2.5 kb V. cholerae DNA segment carrying the mutL gene showing DNA sequencing strategy. The thick line denotes the coding region of the mutL gene. Horizontal arrows show the direction and extent of sequences determined.

To examine the nucleotide sequence homology of V. cholerae mutL and S genes with that of S. typhimurium genes, the 4.3 kb BglII-SalI fragment of the plasmid pGW1811 and 1.54 kb PvuII-HindIII fragment of the plasmid pGW1842 carrying the mutS and mutL genes respectively of S. typhimurium were nick translated and hybridized with EcoRI and PstI digested V. cholerae DNA. Although S. typhimurium genes hybridized with those of V. cholerae, the nucleotide sequence homology was not extensive as judged from the time of exposure to films required and condition of stringency used to visualize the bands in Southern blot hybridization.

Cleavage maps of mutS and mutL genes

The 4.7 kb and the 2.5 kb DNA fragments of V. cholerae complementing mutS and mutL genes of E. coli were eluted from low melting agarose gels and were digested with various restriction endonucleases. The products were analysed by either agarose or polyacrylamide gel electrophoresis. Cleavage maps of the 4.7 kb DNA fragment (Fig. 1) coding for mutS gene product and 2.5 kb fragment (Fig. 2) coding for the mutL function were constructed using several restriction enzymes. Linkages between the restriction fragments were determined either from the analysis of the partial digestion products or from the restriction fragments produced by digestion with two restriction enzymes.

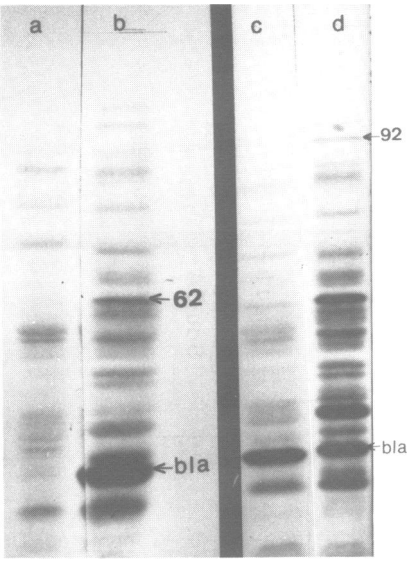

Figure 3. Identification of the mutL and mutS gene products of V. cholerae. UV-irradiated cells of strain CSR603 (lane a) or cells carrying the plasmid pJT250 (lane b), the plasmid pUC8 (lane c) or the plasmid pJT470 (lane d) were labeled with ^{35}S-methionine for 1 hr. and soluble extracts of proteins were prepared as described in Materials and Methods. The proteins were analysed by SDS-polyacrylamide gel electrophoresis followed by autoradiography of dried gels. The numbers represent molecular weight of the polypeptides in kilodaltons. 'bla' represents the β -lactamase gene product.

Digestion of restriction fragments with Bal31 was used to confirm the linkages.

Proteins produced in cells carrying pJT470 and pJT250

The protein(s) coded by the 4.7 kb segment of pJT470 and 2.5 kb segment of pJT250 were examined using maxicell system. E. coli strain CSR603 carrying the plasmids pJT470 and pJT250 were irradiated with ultraviolet light, labeled with ^{35}S-methionine for 1 hr, and soluble extract of protein was prepared as described in Materials and Methods. The extracts were analysed by SDS-polyacrylamide gel electrophoresis and autoradiography. The 2.5 kb and 4.7 kb DNA fragments of V. cholerae complementing E. coli mutL and S gene functions code for proteins of sizes 62,000 and 92,000 dalton respectively (Fig. 3). The level of expression of both mutL and mutS genes were low.

While 50 J/m^2 UV fluence reduced the background of cell protein synthesis in the strain CSR603 (Fig. 3, lane a) and cells carrying the plasmid pJT250 (Fig. 3, lane b), the dose required to reduce the cell protein synthesis in cells

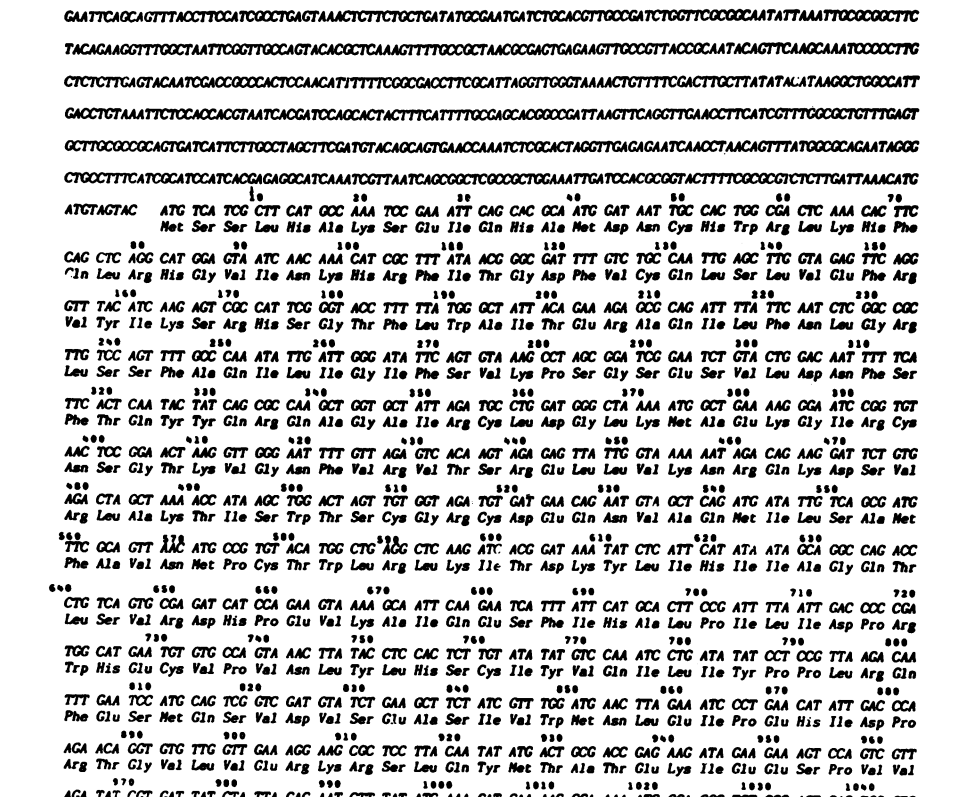

Figure 4. Nucleotide and deduced amino acid sequence of V. cholerae mutL gene and its vicinity.

carrying the plasmid pJT470 was much higher. Even at 80 J/m^2 UV fluence the background of cell protein synthesis was high (Fig. 3, lane d). Whether this is due to the presence of a second gene in the 4.7 kb DNA fragment, which can confer UV resistance to these cells is yet to be determined.

The products of all mutL and mutS genes identified so far in different organisms have molecular weights around 60,000 and 90,000 respectively (1). This close resemblance in size of these proteins and their functional similarities suggest that there might be a common origin of these genes.

Nucleotide sequence of mutL gene

The nucleotide sequence of the 2.5 kb EcoRI fragment of the plasmid pJT250 carrying the mutL gene of V. cholerae has bee determined (Fig. 4). There is only one open reading frame of 563 amino acids that can accomodate the mutL gene product, a 62,000 dalton protein (Fig. 3). Interestingly, the Shine and Dalgarno (SD) sequence of the type -G-G-A-G-G-A- or -A-G-G-, -G-G-A- or -G-A-G- five to nine nucleotides upstream of the initiator codon is not present in the mutL DNA sequence. At -14 position the sequence -T-T-A-A-A- and at -35 position a stretch of thymine is present. The absence of a strong ribosome binding site (RBS) is reflected in the low level of expression of the gene product. It has been reported that the number of molecules per cell of the mutS proteins of S. typhimurium and E. coli are also low (32). The nucleotide sequence of the mutS gene of S. typhimurium does not have any strong homology to promoter consensus sequences as well as RBS immediately upstream of the coding region (32).

ACKNOWLEDGEMENT

We are grateful to Prof. G.C. Walker, U.S.A., for the plasmids and E. coli strains. One of us (TKB) is grateful to the Council of Scientific & Industrial Research for offering a predoctoral research fellowship. The work was supported by the Department of Science & Technology [Grant No. SP/SO/D64/85], Government of India.

*To whom correspondence should be addressed

+Present address: Department of Molecular Biology, The Cleveland Clinic Foundation, Cleveland, OH 44106-4775, USA

REFERENCES

1. Modrich, P. (1987) Ann. Rev. Biochem. 56, 435-466.
2. Radman, M. and Wagner, R. (1986) Ann. Rev. Genet. 20, 523-538.
3. Claverys, J.P. and Lacks, S.A. (1986) Microbiol. Rev. 50, 133-165.

4. Lu, A.L., Clark, S. and Modrich, P. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 4639-4643.
5. Kramer, B., Kramer, W. and Fritz, H.J. (1984) Cell 38, 879-887.
6. Marinus, M.G. and Morris, N.R. (1974) J. Mol. Biol. 85, 309-322.
7. Herman, G.E. and Modrich, P. (1982) J. Biol. Chem. 259, 2605-2612.
8. Guild, W.R. and Shoemaker, N.B. (1976) J. Bacteriol. 125, 125-135.
9. Nevers, P. and Spatz, H.C. (1975) Mol. Gen. Genet. 139, 233-243.
10. Glickman, B.W. and Radman, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1063-1067.
11. Bauer, J., Kramoren, G. and Knippers, K. (1981) Mol. Gen. Genet. 181, 541-547.
12. Su, S.S. and Modrich, P. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 5057-5061.
13. Welsh, K.M., Lu, A.L. and Modrich, P. (1987) J. Biol. Chem. 262.
14. Hickson, I.D., Arthur, H.M., Bramhill, D. and Emmerson, P.T. (1983) Mol. Gen. Genet. 190, 265-270.
15. Kumura, K. and Sekiguchi, M. (1984) J. Biol. Chem. 259, 1560-1565.
16. Leib, M. (1985) Mol. Gen. Genet. 199, 465-470.
17. Jonis, M., Wagner, R. and Radman, M. (1987) J. Mol. Biol. 194, 155-159.
18. Das, G., Sil, K. and Das, J. (1981) Biochim. Biophys. Acta 655, 413-420.
19. Das, G. and Das, J. (1983) Mutat. Res. 109, 21-30.
20. Palit, B., Das, G. and Das, J. (1983) J. Gen. Virol. 64, 1749-1755.
21. Paul, K., Ghosh, S.K. and Das, J. (1986) Mol. Gen. Genet. 203, 58-63.
22. Ghosh, S.K., Panda, D. and Das, J. (1989) Mutat. Res. 210, 149-156.
23. Pang, P.P., Lundberg, A.S. and Walker, G.C. (1985) J. Bacteriol. 163, 1007-1015.
24. Yanisch-Perron, C., Vieira, J. and Messing, J. (1985) Gene 33, 103.
25. Sancar, A., Hack, A.M. and Rupp, D.W. (1979) J. Bacteriol. 137, 692.
26. Roy, N.K., Das, G., Balganesh, T.S., Dey, S.N., Ghosh, R.K. and Das, J. (1982) J. Gen. Microbiol. 128, 1927-1932.
27. Kadonaga, J.T. and Knowles, J.R. (1985) Nucleic Acids Res. 13, 1733-1745.
28. Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning : A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
29. Southern, E.M. (1975) J. Mol. Biol. 98, 503-517.
30. Ray, P., Sengupta, A. and Das, J. (1984) Virology 136, 110-124.
31. Sangar, F., Nicklen, S. and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467.
32. Haber, L.T., Pang, P.P., Sobell, D.I., Mankovich, J.A. and Walker, G.C. (1988) J. Bacteriol. 170, 197-202.