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Genetic Analysis of an Incomplete *mutS* Gene from *Pseudomonas putida*

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We genetically characterized the *Pseudomonas putida mutS* gene and found that it encodes a smaller MutS protein than do the genes of other bacteria. This gene is able to function in the *mutS* mutants of *Escherichia coli* and *Bacillus subtilis*. A *P. putida mutS* mutant has a mutation frequency 1,000-fold greater than that of the wild-type strain.

The MutS protein is part of the MutSLH DNA repair system, which corrects the mismatched DNA produced by DNA replication errors, genetic recombination, and chemical damage to DNA (5). Since MutS proteins are relatively large (~90 kDa), multifunctional proteins, they probably contain multiple domains. Deletion analysis of the *Escherichia coli mutS* gene showed that DNA binding takes place in the N-terminal end of MutS and MutS dimerization and MutS-MutL interaction happen in the C-terminal end (9). Functional analysis of *Thermus thermophilus* MutS showed that it consists of at least three domains, including double-stranded DNA binding, mismatched DNA binding, and APTase (8).

In this study, we identified a *mutS* gene from *Pseudomonas putida* which is able to metabolize various aromatic compounds. We showed that this gene encodes a smaller MutS protein than do genes of other bacteria. We used complementation analysis of this gene in *E. coli* and *Bacillus subtilis*, as well as disruption of the *P. putida mutS* gene.

Cloning and sequencing of a *P. putida mutS* gene. The amino acid sequences of the *E. coli* (7), *B. subtilis* (1), and *Azotobacter vinelandii* (4) MutS proteins share five highly conserved regions in the C-terminal domain. Based on this data, we designed two oligonucleotide primers [forward primer, 5′-GICA TCA(T/C)CCIGTIGTIGTIGA-3′; reverse primer, 5′-TC(A/G)AA (A/G)TA(A/G)TGIGTIG-3′] from two conserved amino acid sequences (GRHPVVE and TLFATHYFELT) and used a PCR with *P. putida* ATCC 33015 chromosomal DNA as the template. A 450-bp DNA segment was amplified and sequenced, which showed that the DNA encodes three conserved amino acid sequences (IITGPNMGGKSTYMRQ, GRSTFMVEM, and SLVLMDE) in the C-terminal domain of MutS. The amplified DNA was used as a probe to screen a cosmid library of the genomic DNA. All of the DNA was partially digested with *Sau*3AI and ligated to *Bam*HI-digested cosmid Lorist6 (Nippon Gene). The ligation mixture was packaged through the use of an in vitro packaging module (Amersham Pharmacia Biotech), and about 3,000 recombinant clones of *E. coli* DH5α were obtained. Southern hybridization screening of 500 clones, in which we used an AlkPhos Direct system for chemiluminescence (Amersham Pharmacia Biotech), yielded three positive colonies. Restriction analysis of these clones showed that all inserts had overlapping regions and each contained a 3.7-kb *Eco*RI-*Hin*dIII fragment which hybridized to the probe (data not shown).

The nucleotide sequence of this fragment was determined, and its genetic organization is shown in Fig. 1. The nucleotide sequence spans 3,726 bp and contains two complete open reading frames of 563 (*orf1*) and 108 (*orf2*) codons. For *orf1*, significant homology to the *Pseudomonas aeruginosa* (accession no. AF220055), *A. vinelandii*, *E. coli*, and *B. subtilis* proteins MutS (82.1, 81.2, 56.8, and 38.9%, respectively) was found. However, *orf1* encoded a smaller protein (about 60 kDa) than that (~90 kDa) of other bacteria. Therefore, to determine whether this gene is a true *mutS* gene or not, we performed, using the method described above, genomic Southern hybridization using the *orf1* fragment as a probe. Our results detected only one band corresponding to the 3.7-kb fragment in the *Eco*RI-*Hin*dIII-digested chromosomal DNA (data not shown). The amino acid sequence of *orf2*, which was located in the same direction as the *mutS* gene, was identical to that of the *fdxA* (ferredoxin) gene from *Pseudomonas ovalis* (3) and was very similar in genetic organization to that of *A. vinelandii* (Fig. 1). Moreover, the N-terminal region (275 amino acids) of the open reading frame, which was located in the direction opposite to the *mutS* gene, was highly homologous to that of the *icd* (isocitrate dehydrogenase) gene from *A. vinelandii* (accession no. D73443). Taken together, the above comparisons suggest that, in spite of an incomplete gene, the small MutS protein of *P. putida* is likely to be structurally and functionally analogous to that of *A. vinelandii*.

Complementation analysis of a *P. putida mutS* gene. To examine the possibility that *P. putida* MutS could complement the *mutS* mutants of other bacteria, we constructed two expression plasmids capable of replicating in *E. coli* and *B. subtilis*. First, we constructed the expression vector pKK223-3M, which modified the multicloning sites of pKK223-3 (Amersham Pharmacia Biotech) by ligating *Hin*dIII multicloning sites of pHSG299 (Takara Shuzo) and an *Eco*RI into them. This created the *Xba*I site. A 1,692-bp fragment of the *P. putida mutS* coding region was amplified by PCR on chromosomal DNA. The forward primer had an *Eco*RI site (5′-GGGAATT CATGGGATACCAGAAAATC-3′; the underlined bases correspond to the *mutS* sequence), and the reverse primer had an *Xba*I site (5′-GGTCTAGATTATAACAGGTTCTTTAG-3′). The fragment was cloned into the *Eco*RI and *Xba*I sites of pKK223-3M. The resulting plasmid, designated pEPPS, was transformed into the BMH71-18 *mutS* mutant strain of *E. coli* (NIG Collection). We also constructed plasmid pEEES by inserting a 2,562-bp fragment of the *E. coli mutS* coding region

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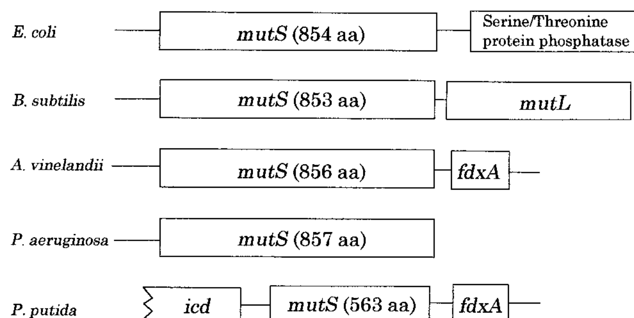

FIG. 1. Genetic organization of the *mutS* gene loci of various microorganisms. aa, amino acid residues.

into the *Eco*RI and *Xba*I sites of pKK223-3M, and this was transformed into the BMH71-18 *mutS* strain and used as a control. For *B. subtilis*, a 1,972-bp *Bam*HI- and *Hin*dIII fragment containing the *tac* promoter and the *mutS* coding sequences of pEPPS was cloned into the *Bam*HI and *Hin*dIII sites of *B. subtilis* plasmid pHY300PLK (Takara Shuzo) and the resulting plasmid, designated pBPPS, was transformed into the 168*trp-S B. subtilis mutS* strain (M. Sasaki and Y. Kurusu, unpublished data).

To investigate whether *P. putida* MutS could complement the *mutS* mutants of both bacteria, we compared the spontaneous mutation rates of these transformants. Through the use of previously described procedures for *E. coli* and *B. subtilis* (1, 2), we measured their frequencies of mutation to rifampin resistance. As shown in Table 1, a *P. putida mutS* gene was partially complemented in an *E. coli mutS* mutant and was completely complemented in a *B. subtilis mutS* mutant. The expression of the *P. putida mutS* gene in both *mutS* mutants was relatively low, since a typical MutS protein could not be detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in either cell (data not shown). These results suggested that the small MutS protein of *P. putida* could function in both bacteria.

Disruption of the *P. putida mutS* gene. To confirm that the incomplete *mutS* gene is a mutator gene in *P. putida*, we constructed a *mutS* mutant of *P. putida* that could not synthesize MutS and compared its spontaneous mutation rate to that of the wild-type strain. We obtained a 1,277-bp internal fragment of the *mutS* gene by digesting plasmid pEPPS with *Sac*I and *Eco*T14I and then treating it with T4 DNA polymerase. By inserting this fragment into the *Hin*cII site of *E. coli* plasmid pHSG299, which contained the kanamycin resistance (Km^r) gene as the selectable marker, we constructed an integrative plasmid. This plasmid was integrated into the chromosomal wild-type *mutS* locus by homologous recombination, which resulted in the plasmid separating two partial-deletion-containing copies of *mutS*. These disruptants were analyzed by Southern hybridization with a suitable probe (data not shown), and one disruptant, designated 33015-S, was used to measure the spontaneous mutation frequency. As shown in Table 1, strain 33015-S had a mutation frequency 1,000-fold greater than that of the wild-type strain. To confirm that the *P. putida mutS* gene could complement the *mutS* mutant of *P. putida*, we constructed plasmid pPPPS by inserting a 1,972-bp *Bam*HI-*Hin*dIII fragment, containing the *tac* promoter and the *mutS* coding sequences of pEPPS, into the *Bam*HI and *Hin*dIII sites of *P. putida* plasmid pSUP104 (6) and transformed them into strain 33015-S. As shown in Table 1, 33015-S carrying pPPPS had a frequency of spontaneous mutation similar to that of wild-type strain 33015. These results indicate that the *mutS* gene is a mutator gene in *P. putida*.

TABLE 1. Spontaneous mutation frequencies of various strains

Strain	Avg frequency of Rif^r cells ± SD[a]	Relative mutation frequency[b]
E. coli		
BMH71-18 *mutS* (MutS$^-$)	$(3.5 \pm 0.4) \times 10^{-6}$	220
BMH71-18 *mutS*(pEEES)	$(7.8 \pm 1.5) \times 10^{-8}$	1
BMH71-18 *mutS*(pEPPS)	$(7.2 \pm 1.3) \times 10^{-7}$	10
B. subtilis		
168*trp* (MutS$^+$)	$(8.7 \pm 1.8) \times 10^{-8}$	1
168*trp-S* (MutS$^-$)	$(2.9 \pm 0.9) \times 10^{-7}$	30
168*trp-S*(pBPPS)	$(4.5 \pm 1.6) \times 10^{-8}$	2
P. putida		
33015 (MutS$^+$)	$(1.3 \pm 0.4) \times 10^{-9}$	1
33015-S (MutS$^-$)	$(1.5 \pm 0.5) \times 10^{-6}$	1,000
33015-S(pPPPS)	$(1.4 \pm 0.5) \times 10^{-9}$	1

[a] All strains grown to the stationary phase in Luria-Bertani medium were inoculated into this medium at approximately 50 cells/ml and grown for 25 generations. Aliquots of the cultures were then diluted and spread on Luria-Bertani plates containing rifampin for the selection of spontaneous mutants. Rifampin was used at the following concentrations: for *E. coli*, 100 μg/ml; for *B. subtilis*, 30 μg/ml; for *P. putida*, 50 μg/ml. Cell concentration was determined after the growth of 25 generations. To calculate the standard deviation, all of the experiments were repeated at least five times. Frequencies were calculated from both the total number of cells and the number of Rif^r cells.

[b] Relative frequencies were obtained by comparison with BMH71-18 *mutS*(pEEES) as 1 for *E. coli*, 168*trp* (MutS$^+$) as 1 for *B. subtilis*, and 33015 (MutS$^+$) as 1 for *P. putida*.

Nucleotide sequence accession number. The nucleotide sequence described here has been deposited in the DDBJ/GenBank/EMBL database under accession no. AB039965.

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