

Short Communication

Genetic analysis of *Bacillus subtilis* mutator genes

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(Received March 22, 2000; Accepted June 23, 2000)

Key Words—*Bacillus subtilis*; gene disruption; mutator

Mutators are useful tools for elucidation of the mechanism that controls the spontaneous mutation rate of cells. In the case of *Escherichia coli*, various types of mutators have been isolated and genetically characterized. The MutT protein specifically degrades 8-oxo-dGTP to 8-oxo-dGMP to eliminate them from the nucleotide pool of the oxidized form of guanine (Maki and Sekiguchi, 1992). The MutM enzyme is an N-glycosylase that specifically removes the 8-oxo-guanine base from the deoxyribose sugar in DNA when 8-oxo-dGTP has been mistakenly incorporated in DNA (Tchou et al., 1991). The MutY N-glycosylase specifically removes the adenine base that might have been mistakenly incorporated opposite an 8-oxo-guanine base in DNA (Au et al., 1989). The methyl-directed mismatch repair system composed of MutS, MutL, and MutH protein corrects mismatched DNA produced by DNA replication errors, genetic recombination, and chemical damage to DNA (Modrich, 1989).

Recently, the complete genome sequence of *Bacillus subtilis*, the best-characterized bacterium among the Gram-positive bacteria, has been determined, and many putative mutator genes have been identified (Kunst et al., 1997). The amino acid sequence homologies of these gene products with those of *E. coli*

are relatively low, as follows: MutT, 27.4%; YjhB (MutT homologue), 42.9%; Yvcl (MutT homologue), 32.7%; MutM, 38.8%; YfhQ (MutY homologue), 38.0%; MutS, 40.2%; MutL, 30.7%. However, no studies have been made on the genetic analysis of *B. subtilis* mutator genes except for the *mutSL* operon (Ginetti et al., 1996). In the present study, we analyze these *B. subtilis* genes by using the method of gene disruption and compare them in regard to the frequency of spontaneous mutations with *E. coli*. The goal of the study was to construct the hyper mutator strain of *B. subtilis* and use it to induce in vivo mutagenesis.

Gene disruption of various mutator genes in *B. subtilis*. Various disruptants were constructed by gene replacement employing double crossover and insertion of antibiotic-resistant genes. A PCR amplification of the target gene, using the chromosomal DNA of *B. subtilis* 168 (*Bacillus* Genetic Stock Center, Ohio State University, OH, USA) as a template and primers (shown in Table 1), was run for 30 cycles under the following conditions: denaturation, 1 min at 98°C for the first cycle and 10 s at 98°C thereafter; annealing, 1 min at 55°C; extension, 1 min at 72°C. The amplified DNA was digested with appropriate restriction enzymes (shown in Table 1) and cloned into the *E. coli* plasmid pHSG399 (Takara Shuzo, Tokyo, Japan). Each integrative plasmid was constructed by the insertion of an appropriate antibiotic-resistant cassette into the target gene of the following plasmids: erythromycin-resistant gene from pDG647; tetracycline-

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Table 1. Nucleotide sequence of synthetic primers used for PCR.

Mutator gene	Sequence of primer (5'→3'; forward and reverse)	Restriction site ^a
<i>mutT</i>	AAGGTACC AC CTGGCGAGAGACCAGTA -990	<i>KpnI</i>
	AAAAGCTT GT CTGAATCGAGCTGTATC 1,410	<i>HindIII</i>
<i>yjhB</i>	AACCCGGG AG GTCTAATGGACTAGTAA -985	<i>SmaI</i>
	AACCCGGG TCC ATGTTAATGATGGAAT 1,470	<i>SmaI</i>
<i>yvcl</i>	AACCCGGG AC AGACGGCGTATTCATTT -575	<i>SmaI</i>
	AACCCGGG TCG GCTAATGTCACAGAGC 1,310	<i>SmaI</i>
<i>mutM</i>	AAGAATTC AT GGATGTGCCGGAATTACCA 1	<i>EcoRI</i>
	AAAAGCTTT CA CTTTTTTGTCTGGCACTTT 834	<i>HindIII</i>
<i>yfhQ</i>	AAGTTAAC AG AAAGCATGTGCTGTCGT -979	<i>HpaI</i>
	AAGTTAAC CG CGCATCCTCAAGCAGA 1,453	<i>HpaI</i>
<i>mutS</i>	AAGAATTC AT GATACAGCAATATTTAAAA 1	<i>EcoRI</i>
	AAGCATGC TTA ATGTAATTTCTTTGCAGC 2,574	<i>SphI</i>
<i>mutL</i>	AAGTTAAC GT GGCAAAAGTCATCCAACGT 1	<i>EcoRI</i>
	AAGCATGC CTA CATCACGCGTTTGAACATC 1,881	<i>SphI</i>
<i>nth</i>	AAGTTAAC AA TCGCGTCTTAAAGAACA -1,000	<i>HincII</i>
	AAGTTAA CTC AGGATGATACACAAACT 1,539	<i>HincII</i>

^a The restriction site for cloning has been underlined in the sequence.

Numbers shown on primer sequences (bold type) are indicated in terms of the distance (bp) from initiation codon.

resistant gene from pDG1515; spectinomycin-resistant gene from pDG1726. All plasmids were obtained from *Bacillus* Genetic Stock Center, Ohio State University, and all were treated with appropriate restriction enzymes and T4 DNA polymerase. The composite plasmid was transformed into *B. subtilis* 168 selected for antibiotic resistance and sensitivity at the following concentrations: chloramphenicol, 5 µg/ml; erythromycin, 0.5 µg/ml; tetracycline, 10 µg/ml; spectinomycin, 100 µg/ml. As shown in Fig. 1, all the disruptants used in this study were confirmed by PCR amplification with suitable primers at the following conditions: denaturation, 1 min at 98°C for the first cycle and 10 s at 98°C

thereafter; annealing, 1 min at 55°C; extension, 3 min at 72°C.

Frequencies of spontaneous mutation of various disruptants. The frequency of spontaneous mutation of an *E. coli mutT* mutant was shown to be 1,000–10,000 times greater than that of the wild-type strain (Maki and Sekiguchi, 1992). In *B. subtilis*, three *mutT* genes including the two homologues have been reported (Kunst et al., 1997). To analyze the role of these genes, we constructed four disruptants in which MutT could not be synthesized (Fig. 1). We found that each of three disruptants was not affected in terms of the frequency of spontaneous mutations (Table 2). More-

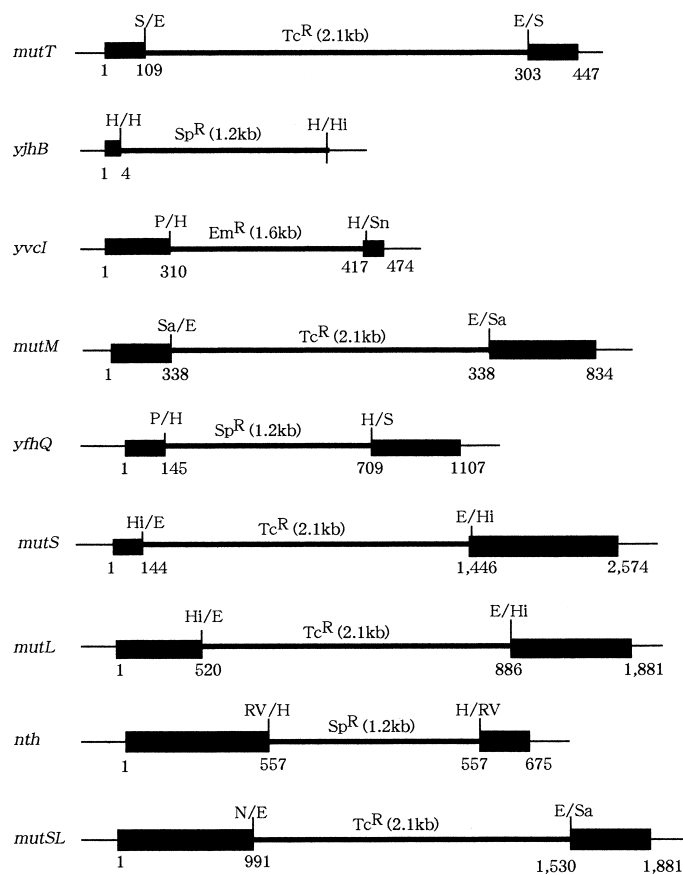


Fig. 1. Schematic representation of the relevant part of the disruptant chromosome.

A general procedure of gene disruption was described in the text. The insertion of antibiotic-resistant cassettes was used for the following genes: Tc^R cassette (2.1 kb) for *mutT*, *mutM*, *mutS*, *mutL*, and *mutSL*; Sp^R cassette (1.2 kb) for *yjhB*, *yfhQ*, and *nth*; Em^R cassette (1.6 kb) for *yvcl*. Numbers are shown on coding sequences (black box) from the initiation codon as 1 to stop codon. Restriction sites are S, *Sma*I; E, *Eco*RI; H, *Hinc*II; P, *Pst*I; Sn, *Sna*BI; Sa, *Sac*I; RV, *Eco*RV; N, *Nsp*V.

over, the same result was obtained by a triple disruptant of these genes (Table 2). These results suggested that these gene products might not be MutT proteins and that other chromosome genes to suppress the synthesis of 8-oxo-dGTP might exist in the *B. subtilis*.

The *mutM* and *mutY* mutants of *E. coli* cells have been shown to be 10- and 100-fold high frequency of spontaneous mutation, respectively, that did the wild-type strain (Au et al., 1989; Tchou et al., 1991). The effects of double mutations in both genes were additive, not what they would have been if only one was mutated (Michaels et al., 1992; Tajiri et al., 1995). For comparison with *E. coli*, we constructed three disruptants in which MutM, MutY, and both could not be synthesized, respectively (Fig. 1). Although two disruptants of *mutM* and *yfhQ* (*mutY* homologue) were relatively affected in regard to the frequency of sponta-

neous mutations, a double disruptant of both genes was shown to be 1,000-fold greater than the wild type (Table 2). These results suggested that the both genes of *B. subtilis*, as well as those of *E. coli*, could function cooperatively. It was concluded that the gene product of *yfhQ* genetically acts as a MutY protein.

The MutSLH system of *E. coli* could act in the newly synthesized strand during mismatch repair at the hemimethylated GATC site (Modrich, 1989). However, the *mutH* gene, which encodes the endonuclease to interact with methylated DNA, is missing in the *B. subtilis* genome because of the absence of known methylation in this bacteria (Kunst et al., 1997). Therefore we searched the highly homologous protein with *E. coli* MutH among the *B. subtilis* genes encoding endonuclease and thought that the most probable candidate gene, 16.5% identical to *E. coli* MutH, was the *nth*

Table 2. Frequencies of spontaneous mutation of various disruptants.

Disrupted gene	Frequency of Rif ^R cells \pm SD ^a	Relative frequency ^b
<i>mutT</i>	$(3.3 \pm 0.2) \times 10^{-9}$	1
<i>yjhB</i>	$(3.4 \pm 0.3) \times 10^{-9}$	1
<i>yvcl</i>	$(2.0 \pm 0.4) \times 10^{-9}$	1
<i>mutT, yjhB, yvcl</i>	$(1.6 \pm 0.2) \times 10^{-9}$	1
<i>mutM</i>	$(1.7 \pm 0.3) \times 10^{-8}$	5
<i>yfhQ</i>	$(3.1 \pm 0.7) \times 10^{-7}$	100
<i>mutM, yfhQ</i>	$(3.3 \pm 0.5) \times 10^{-6}$	1,000
<i>mutS</i>	$(6.8 \pm 1.3) \times 10^{-7}$	226
<i>mutL</i>	$(2.4 \pm 0.8) \times 10^{-7}$	80
<i>nth</i>	$(1.7 \pm 0.2) \times 10^{-8}$	5
<i>mutS, mutL</i>	$(5.9 \pm 1.1) \times 10^{-7}$	196
<i>mutS, mutL, nth</i>	$(8.6 \pm 1.4) \times 10^{-7}$	286
Wild type	$(3.1 \pm 0.2) \times 10^{-9}$	1

^a *B. subtilis* cells grown to the stationary phase in LB medium were inoculated into the same medium at approximately 50 cells/ml and grown for about 25 generations. Aliquots of the cultures were then diluted and spread on LB plates containing 20 μ g/ml of rifampicin for the selection of spontaneous mutants. Cell concentration was determined after the growth of 25 generations. To calculate the standard deviation, experiments were repeated at least five times. Frequencies were calculated from both numbers of total cells and Rif^R cells.

^b Relative frequencies were obtained by comparison with the wild type as 1.

gene encoding endonuclease III (Kunst et al., 1997). To analyze this gene, we constructed five disruptants, including *mutS*, *mutL*, and *nth* genes (Fig. 1). A double disruptant of *mutS* and *mutL* genes was shown to have the same frequency of spontaneous mutations as each of two disruptants of *mutS* and *mutL* (Table 2). An *nth* disruptant was slightly affected, and a triple disruptant of three genes was shown to induce the same results as those described previously in *E. coli* (Modrich, 1989). These results suggested that the *nth* gene might be involved in at least the DNA repair system. We find these results interesting, especially in regard to whether the gene product of *nth* is a MutH protein, and we are currently exploring these issues further.

A JH642 strain has a chromosomal deletion including the *mutT* gene. First we used JH642 chromosomal DNA as a template for the PCR amplification of mutator genes, with only a *mutT* gene being unable to be amplified. A previous report showed that a JH642 strain had a deletion of about 20 kb in size in the chromosome on the genetic map from 32.6° to 42.1° (Itaya, 1993). Therefore to determine the deleted region in the chromosome of a JH642 strain, PCR amplification was done by using various primers. A 1.3 kb fragment DNA was amplified by using the following primers: the forward primer in the *ydzA* gene,

5' CTTGATGTTCTAGATCACCA3' and the reverse primer in the *ydaT* gene, 5' GGATTATCCTGCTTCA-TT3'. The nucleotide sequence of the amplified DNA revealed that a 16,817 bp fragment containing the *mutT* and 12 genes was deleted from 474,925 to 491,742 bp in the chromosome of the *B. subtilis* 168 strain. Moreover, we examined the spontaneous mutation frequency of a JH642 strain by using the same method as that described in Table 2. A JH642 strain was quite similar to a 168 strain in regard to the frequency of spontaneous mutations (data not shown). Taken together, the above results strongly indicated that a *mutT* could not genetically act as a mutator gene in *B. subtilis*.

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