Characterization of a dam Mutant of Serratia marcescens and Nucleotide Sequence of the dam Region

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The DNA of Serratia marcescens has N₆-adenine methylation in GATC sequences. Among 2-aminopurine-sensitive mutants isolated from S. marcescens Sr41, one was identified which lacked GATC methylation. The mutant showed up to 30-fold increased spontaneous mutability and enhanced mutability after treatment with 2-aminopurine, ethyl methanesulfonate, or UV light. The gene (dam) coding for the adenine methyltransferase (Dam enzyme) of S. marcescens was identified on a gene bank plasmid which alleviated the 2-aminopurine sensitivity and the higher mutability of a dam-13:Ts mutant of Escherichia coli. Nucleotide sequencing revealed that the deduced amino acid sequence of Dam (270 amino acids; molecular mass, 31.3 kDa) has 72% identity to the Dam enzyme of E. coli. The dam gene is located between flanking genes which are similar to those found to the sides of the E. coli dam gene. The results of complementation studies indicated that like Dam of E. coli and unlike Dam of Vibrio cholerae, the Dam enzyme of S. marcescens plays an important role in mutation avoidance by allowing the mismatch repair enzymes to discriminate between the parental and newly synthesized strands during correction of replication errors.

In Escherichia coli, the Dam enzyme (DNA adenine methyltransferase) catalyzes the methylation of adenine at N₆ in the sequence GATC in duplex DNA (20, 21), a reaction in which S-adenosylmethionine is the methyl group donor and also an allosteric effector (7). The methylation occurs with a delay at replication forks and prevents the newly synthesized DNA strand temporarily unmethylated (6, 12). The first dam mutants of E. coli were isolated by Marinus and his coworkers (27, 28). From studies with such mutants and strains which overproduce the Dam enzyme, several important roles of the methylation status of DNA have been deduced. First, dam hemimethylation of DNA at the replication fork is required for parental strand-sequence in tion (32, 40). Finally, the methylation status of GATC sequences in the origin region is critical for replication (31, 39, 45) and are hypermutable by and sensitive to various mutagens.

In S. marcescens, we have isolated a dam-13 mutant of S. marcescens. Here we describe some of its properties with respect to spontaneous and induced mutability. We have also identified, cloned, and sequenced a DNA fragment of S. marcescens with the dam gene and neighboring sequences. Our results suggest that the dam gene of S. marcescens differs in structure and function from the V. cholerae gene and is more like those of E. coli and S. typhimurium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The strains and plasmids used are listed in Table 1. If not stated otherwise, TBY broth medium (10 g of Bacto Tryptone, 5 g of Bacto Yeast Extract, 5 g of NaCl per 1,000 ml) was used. TBY plates contained 15 g of Difco agar per 1,000 ml. The incubation was at 30°C. If required, the media contained antibiotics or other supplements as detailed later in the text.

Bacterial transformations. S. marcescens cells were transformed by the Ca²⁺ method as described by Takagi and Kusumi (44), and E. coli cells were transformed by the method of Hanahan (19). dam mutants of E. coli and S. marcescens were transformed by electroporation. Electrocompetent cells were prepared

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from log-phase cultures (1 × 10^8 to 2 × 10^8 cells/ml) as described by Dower et al. (14) and stored at −80°C in 10% glycerol. For electroporation (14) employing a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.), the parameters were 12,500 V/cm, a capacitance of 25 µF, and a parallel resistance of 200 Ω.

**Determination of spontaneous mutation frequencies.** Overnight cultures in TBY (5 ml) were started from single colonies. After 18 h of growth, the cultures were diluted in phosphate buffer and samples were plated after appropriate dilution on selective media (TBY with chloramphenicol, tetracycline, kanamycin, ampicillin, or rifampin) and on TBY (viable count). The mutation frequency is the ratio of the resistant-cell titer to the viable-count titer.

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**Treatment of cells with UV, 2-AP, or ethyl methanesulfonate (EMS).** The following experiments were done at least in triplicate with cultures each started from a single colony.

(i) UV. Log-phase cells grown in TBY were resuspended in phosphate buffer (10 ml, 2 × 10^8 cells/ml) and irradiated with a UV dose of either 0, 3, 6, or 9 J/m^2 (dose rate, 0.6 J/m^2 s). Samples of 0.1 ml were diluted in phosphate buffer and plated for measurement of survival. The remaining cells were sedimented, resuspended in TBY, and aerated at 30°C for 4 h. The cells were then sedimented and resuspended in 1 ml of TBY. Undiluted or appropriately diluted samples were then plated on selective (TBY with chloramphenicol or rifampin) and nonselective (viable count) media to determine the frequencies of resistant mutants.

(ii) 2-AP. Stationary-phase cells grown in M9 medium (26) were diluted in M9 medium to give a titer of 2 × 10^8 cells/ml. To 10 ml samples, 2-AP from an aqueous stock solution (5 mg/ml) was added to final concentrations of 0, 10, 50, and 100 µg/ml. The cultures were aerated at 30°C until the sample without 2-AP reached a titer of 4 × 10^8 cells/ml (about 3 h). The cells of all samples were then sedimented, washed twice in phosphate buffer, and each resuspended in 1 ml of phosphate buffer. Appropriate dilutions were plated on selective media (TBY with chloramphenicol or rifampin) and on TBY (viable count) to determine the frequencies of resistant mutants.

(iii) EMS. EMS was added to 10-ml samples of a log-phase culture grown in TBY to give final concentrations of 0, 0.2, 0.5, and 1 µg/ml. After 10 min at 30°C in a shaking incubator, the cells were sedimented and washed with TBY and samples were plated after appropriate dilution on TBY to determine survival. Of the remaining suspensions, 2.5 ml was added to prewarmed TBY and incubated for 4 h at 30°C. The cells were then sedimented and resuspended in 1 ml of TBY, and appropriately diluted samples were plated on selective (TBY with chloramphenicol or rifampin) and nonselective (viable count) media to determine the frequencies of resistant mutants.

**Nucleotide sequence accession number.** The sequence described in this report has been deposited in the EMBL database under accession no. X78412.

**RESULTS**

Isolation of a dam mutant. Preliminary experiments had shown that chromosomal DNA of *S. marcescens* Sr41 and pBR322 DNA isolated from a plasmid-bearing derivative of this strain were cleaved in vitro by restriction endonuclease Sau3AI and were refractory to digestion with MboI. Both enzymes recognize the sequence GATC. Sau3AI cleaves it irrespective of methylation at A, whereas MboI cleaves only the nonmethylated sequence. Corresponding to previous studies on DNA methylation by restriction analysis (15), it was concluded that in *S. marcescens* Sr41, the GATC sequences are methylated as in *E. coli* by the Dam enzyme (28). This corresponds to a previous finding of dam methylation in *S. marcescens* (5). Since it is known that dam mutants of *E. coli* are sensitive to 2-AP (17), we decided to use 2-AP sensitivity in screening for a dam mutant of *S. marcescens*. In a sensitivity test with *S. marcescens* Sr41, it was found that 1,000 µg of 2-AP/ml of TBY agar was the highest concentration which did not suppress the growth of the strain. About 3,000 colonies from a mutagenized culture were replica plated on 2-AP agar (1,000 µg/ml) and six 2-AP-sensitive clones were identified. The six mutants were found to have resistance to UV irradiation and mitomycin C treatment (growth on TBY agar with 1 µg of mitomycin C/ml) similar to that of the parental strain, indicating that they were not recA mutants, which are known to be highly sensitive to these DNA-damaging agents. After all six 2-AP-sensitive strains were transformed with pBR322, the plasmid DNA isolated from only one of the mutants was cleavable by both Sau3AI and MboI. This indicated that the mutant lacked dam-specific DNA methylation. The organism was termed the dam-1 mutant and characterized further.

**Characterization of the dam-1 mutant.** In *E. coli*, the deficiency of dam activity results in increased mutability. To examine the phenotype of the *S. marcescens* dam-1 mutant, the frequencies of various spontaneous forward mutations were determined (Table 2). Compared to the wild type, the frequencies of mutation to rifampin resistance (Rif^r^), chloramphenicol resistance (Cm^r^), and ampicillin resistance (Ap^r^) were increased about 8-fold. No strong increase in the frequency of tetracycline resistance (Tc^r^) was observed (Table 2). Hypermutability of the dam-1 strain was also found when the frequencies of induced mutations (Rif^r^ and Cm^r^) were determined after treatment of cells with various mutagens. The dam-1 strain was slightly more UV sensitive than the wild type, and the number of induced Rif^r^ and Cm^r^ mutants increased much more with increasing UV doses in the dam-1 strain than in the wild type (Fig. 1a). In the presence of 0.5% EMS, the survival of the dam mutant was 32% (wild type, 90%)

### TABLE 1. Bacterial strains and plasmids used in this study

<table>
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<tr>
<th>Strain or plasmid</th>
<th>Size (kb)</th>
<th>Relevant genotype or alternate designation</th>
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<td>K-12s</td>
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<td>19</td>
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<td>dam-13: Tn9; Cm^r^</td>
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<td>This work</td>
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*dam* GENE OF *S. MARCESCENS* 3881
and the frequency of Rif\(^r\) was \(3 \times 10^{-6}\) (wild type, \(1 \times 10^{-6}\)). A result similar to that obtained with EMS was also obtained with 2-AP (Fig. 1b). The remarkable sensitivity of the \(dam^{-1}\) strain to 2-AP parallels observations with \(E. coli\) and \(S. typhimurium\) (17, 39) and allowed the isolation of the mutant. The increased spontaneous and induced mutability of the \(dam^{-1}\) strain suggests that a mechanism of mutation avoidance is blocked, a phenotype also seen in \(E. coli\) \(dam\) mutants (17).

Cloning of the \(dam\) gene. Since the expression of the \(dam^+\) gene provides resistance of cells to 2-AP in \(S. marcescens\) and \(E. coli\), we used this phenotype to screen a monocopy cosmid genomic library of \(S. marcescens\) (13) for a plasmid carrying the \(dam\) gene. For this, the \(E. coli\) \(dam^{-13}\) mutant was transformed with the library cosmids (selection was for Ap\(^r\)) and the transformant colonies were replica plated on TBY supplemented with 900 \(\mu\)g of 2-AP/ml. At this concentration, the \(E. coli\) \(dam^{-13}\) strain no longer grew. Several 2-AP-resistant transformants were identified. Their plasmids were isolated and tested for \(dam\)-specific DNA methylation by treatment with MboI and Sau3AI, respectively. The DNA of all plasmids was restricted with Sau3AI and not by MboI, indicating a \(Dam^+\) phenotype of the cells. The plasmid of one of the transformants was termed pTO1B. Its restriction map (Fig. 2) indicated that it contained a 27.5-kb insert of DNA.

Restriction fragments of the insert of pTO1B were subcloned into pBR328 by using \(E. coli\) DH5\(\alpha\). Lack of cleavage by MboI of a plasmid with the 3.2-kb ClaI fragment after passage through \(E. coli\) dam-13 indicated that the \(dam\) gene was expressed from this fragment (pTO3C; Fig. 2).

Nucleotide sequence of the \(dam\) region. Overproduction of the Dam enzyme results in a hypermutable phenotype in \(E. coli\). Therefore, the DNA for sequencing of the \(dam\) region was prepared from Bluescript multicopy plasmids in which only parts of the \(dam\) gene were cloned. As shown in Fig. 2, these plasmids were pTO6A (1.35-kb EcoRI-SacI fragment) and pTO11A (1.5-kb BamHI-ClaI fragment), both of which did not provide Dam activity (data not shown). The sequences of both inserts were determined and aligned. The 2,340 bp revealed three open reading frames (ORFs) of 642, 810, and 630 bp respective to that deduced from the \(urf\) 74.3-nucleotide sequence of \(E. coli\) which is located in front of \(dam\) (22). The second ORF codes for a protein of 270 amino acids (aa) (molecular mass, 31.3 kDa) with 72% sequence identity to the \(E. coli\) Dam protein (278 aa; 10, 22, 24). This gene was termed \(dam\) of \(S. marcescens\). The amino acid sequence encoded by the \(dam\) gene contains motifs I to VIII and X, which are conserved among members of the \(\alpha\) group of \(N_6\)-adenine

and the events of Rif\(^r\) mutant numbers with increasing 2-AP doses...
The previously described dam mutant of another member of the γ subdivision of Proteobacteria, V. cholerae, remains somewhat of an enigma. The mutant is sensitive to 2-AP, methyl methanesulfonate, and UV but retains methylation of GATC sequences and normal mutability (4). The cloned gene, when highly overexpressed from a multicopy plasmid, can complement an E. coli dam mutant (alleviation of UV and 2-AP sensitivity and increased spontaneous mutability) but does not increase the spontaneous mutability of E. coli or V. cholerae (3, as seen with the overexpressed Dam enzymes of E. coli (21, 30) and S. marcescens (Table 3). The enzyme is relatively small (192 aa) and has no significant amino acid sequence identity with the Dam enzyme of E. coli or S. marcescens. Perhaps the described Dam of V. cholerae is part of a restriction-modification system and exists in V. cholerae in addition to another Dam enzyme related to mismatch correction. How lack of the identified Dam in V. cholerae would interfere with DNA repair remains unclear.

Spontaneous mutability to resistance to various antibiotics was differentially increased in the dam mutant of S. marcescens. Whereas the Rif’ mutation frequency was elevated about 30-fold, the Te’ frequency was not different from that of the wild type (Table 2). It is possible that GATC sequences are not present in the corresponding chromosomal region or that existing GATC sequences are specifically not methylated (18, 38) so that methyl-directed mismatch repair is inefficient in that region. Sites with GATC refractory to complete methylation have been identified on the E. coli chromosome (38). This would be consistent with the already high Te’ mutation frequency in wild-type cells and suggests that lack of methylated GATC sequences in that region would not keep mutability down. In the genome of E. coli, the frequency of GATC sequences is correlated with the efficiency of mismatch repair (23) and the probability of repair of a mismatch decreases with the distance of the mismatch from the next GATC site (11). In this context, it is interesting that overexpression of dam’ causes a five- to sixfold higher spontaneous mutation frequency than that resulting from dam’ deficiency (Table 3). Possibly, when dam is overexpressed, mismatch correction is close to zero. Compared to this, in wild-type cells, about 99% of replication errors are corrected, and in dam-deficient cells, substantial mismatch correction by the mutH LS system is still apparent (Table 3). This would be consistent with previous studies (30) and with the finding that MutH, which is the nuclease component of the mismatch repair system, cleaves DNA when hemimethylated or nonmethylated but not when fully methylated (1, 48). Another explanation for the lower
mutability of dam mutants than that of dam−-overexpressing strains could be that frequent MutHLS-caused double-strand breaks leave mutants inviable (35).

A phenotypic difference between the dam mutant of S. marcescens and those of E. coli was observed with respect to UV-induced mutability. Much higher numbers of mutants relative to the wild type were induced per UV dose in the dam mutant strain of S. marcescens compared to the corresponding numbers in E. coli (Fig. 1; 17). Glickman et al. (17) postulated that the methyl-directed mismatch repair system would reduce direct mutagenesis resulting from wrong single-base incorporations during replication but not indirect mutagenesis caused by nonpairing DNA lesions like pyrimidine dimers, which obstruct DNA synthesis. Such lesions would lead to mutation via SOS induction of the error-prone repair pathway. However, the repair of UV damage in DNA requires extensive repair replication (46) in the form of short patches (12 bases per lesion) or long patches (up to 2,000 bases per lesion). Therefore, in a dam mutant, misincorporations during repair replication would also lead to direct mutagenesis. If the contributions to mutation avoidance of proofreading by DNA polymerase and of mismatch correction were more on the side of mismatch correction in S. marcescens than in E. coli, then a higher mutability of the dam mutant of S. marcescens after DNA damage requiring repair replication could be expected, as was found. Alternatively, the repair after UV irradiation could more frequently involve long-patch repair in S. marcescens than in E. coli.

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REFERENCES

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