Spontaneous Mutations Occur Near Dam Recognition Sites in a *dam*$^-$ *Escherichia coli* Host

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ABSTRACT

The mismatch repair system of *Escherichia coli* K12 removes mispaired bases from DNA. Mismatch repair can occur on either strand of DNA if it lacks N^6-methyladenines within 5′-GATC-3′ sequences. In hemimethylated heteroduplexes, repair occurs preferentially on the unmethylated strand. If both strands are fully methylated, repair is inhibited. Mutant (*dam*$^-$) strains of *E. coli* defective in the adenine methylase that recognizes 5′-GATC-3′ sequences (Dam), and therefore defective in mismatch repair, show increased spontaneous mutation rates compared to otherwise isogenic *dam*$^+$ hosts. We have isolated and characterized 91 independent mutations that arise as a consequence of the Dam$^-$ defect in a plasmid-borne phage P22 repressor gene, *mnt*. The majority of these mutations are A:T→G:C transitions that occur within six base pairs of the two 5′-GATC-3′ sequences in the *mnt* gene. In contrast, the spectrum of *mnt*$^-$ mutations in a *dam*$^+$ host is comprised of a majority of insertions of IS elements and deletions that do not cluster near Dam recognition sites. These results show that Dam-directed post-replicative mismatch repair plays a significant role in the rectification of potential transition mutations *in vivo*, and suggest that sequences associated with Dam recognition sites are particularly prone to replication or repair errors.

THE *dam* (*D*NA *a*denine *m*ethylation) gene of *Escherichia coli* encodes an enzyme, Dam, that methylates the 6-amino group of adenine in the recognition sequence, 5′-GATC-3′ (for review, see MARINUS 1984, 1987). Although Dam is not essential for the growth of *E. coli*, it is thought to be required for strand discrimination during mismatch repair both *in vivo* and *in vitro* (PUKKILA *et al.* 1983; LU, CLARK and MODRICH 1983). Mismatch repair occurs after replication on a substrate in which a nascent, undermethylated daughter strand is paired with a fully methylated parental strand. Differential methylation presumably allows the discrimination between daughter and parent strands so that mismatched bases are removed preferentially from the daughter strand (CLAVERYS and LACKS 1986; MESELSON 1987; MODRICH 1987).

In a *dam*$^-$ mutant, neither DNA chain is methylated. In such a host, strand discrimination should be lost. For a given mismatch, removal of the base from the parental strand should occur as frequently as from the daughter strand. Consistent with this idea, *dam*$^-$ strains exhibit higher spontaneous mutation rates than otherwise isogenic *dam*$^+$ strains (MARINUS and MORRIS 1974). If these higher rates are due to the failure of *dam*$^-$ hosts to rectify mispairs arising from misincorporation events, then the spectrum of mutations in *dam*$^-$ host should represent these events.

To investigate what spectrum of mutations arises in a *dam*$^-$ host, we selected for mutations in a small target gene, *mnt*, on plasmid pMQ151. This plasmid carries the tetracycline-resistance determinant (*tetA*) from plasmid pBR322 under the negative control of *Salmonella* phage P22 Mnt repressor (Figure 1). Mutations that inactivate the *mnt* gene or its operator permit the constitutive transcription of the *tet*A structural gene from the phage *ant* promoter in this operon fusion, and enable the plasmid to confer a tetracycline-resistant phenotype upon a sensitive host (YOUDERIAN *et al.* 1983). Since the *mnt* gene is small (252 base pairs), and the Mnt operator is close to the *mnt* gene (within 58 base pairs), a large number of independent mutations that inactivate either target can be sequenced rapidly.

Previously, we described the isolation and characterization of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG)-induced mutations in the *mnt* repressor gene (LUCCHESI, CARRAWAY and MARINUS 1986). We found that 29 of 30 MNNG-induced mutations in the *mnt* gene were G:C→A:T transitions and one was an A:T→G:C transition, consistent with the findings of other studies (COULONDRE and MILLER 1977; LOECHLER, GREEN and ESSIGMAN, 1984). In this report, we use the *mnt* repressor gene as a target to determine the nature of DNA sequence changes induced in a *dam*$^-$ strain. We find the surprising result that the majority of spontaneous *mnt*$^-$ mutations in a *dam*$^-$ genetic background are A:T→G:C transition mutations clustered near Dam recognition sites.

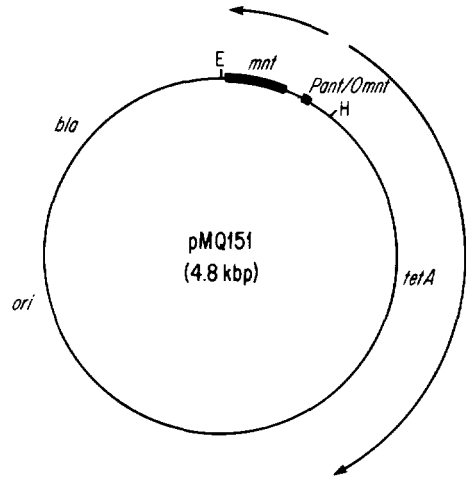

FIGURE 1.—The P_{ant}/*tetA* operon fusion. The *immI* region of phage P22 DNA subcloned in pBR322 is indicated. E and H indicate the *Eco*RI and *Hin*dIII sites of pBR322, respectively. Mnt repressor binds to O_{mnt}, preventing rightward transcription of the *tet*A gene from P_{ant} (indicated by the long arrow). The *mnt* gene is transcribed in the opposite direction. Plasmid pMQ151 carrying this fusion operon confers a tetracycline-sensitive phenotype.

MATERIALS AND METHODS

Bacterial strains and plasmids: *E. coli* strain 1200 (F⁻ *end-1 rns-1 thi-1 rel-1 supE44*) and its *dam-3* derivative GM215, were transformed with plasmid pMQ151 to give strains GM3133 and GM3106, respectively. Plasmid pMQ151 (LUCCHESI, CARRAWAY and MARINUS 1986) contains a 500 bp portion of the *immI* region of *Salmonella* phage P22 (Fig. 1). Strains MM294 (F⁻ *endA1 thi1 hsdR17 supE44*) and 1200 were obtained from the *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven, CT 06510.

Isolation of tetracycline-resistant strains: Dilutions containing several hundred cells from an overnight culture were inoculated into 1 ml of brain-heart infusion (BH) plus 40 μg/ml of ampicillin, and grown overnight at 37°. Aliquots (0.1–0.3 ml) of the overnight cultures were spread on BH plates containing 20 μg/ml ampicillin and 3.5 μg/ml tetracycline, and incubated at 37°. A single colony from each plate was purified on media with 10 μg/ml tetracycline. Plasmid DNAs were extracted (DAVIS, BOTSTEIN and ROTH 1980) and used to transform strain MM294 to a tetracycline-resistant phenotype (BECKINGHAM and WHITE 1980). This extra step was necessary because we found that plasmid DNAs isolated from *dam-3 end-1* strains are not suitable substrates for enzymatic sequence analysis using *E. coli* DNA polymerase I large fragment. Synthetic products of sequencing reactions were obscured by high levels of background incorporation of radioactive label into DNA fragments of all sizes. We speculate that this is due to an abundance of transient strand breaks in DNA isolated from a *dam*⁻ host, but not in an otherwise isogenic *dam*⁺ host.

Preparation of plasmid DNA and DNA sequencing: Plasmid DNA was purified as described by BIRNBOIM and DOLY (1979), except that two phenol extractions were included. Supercoiled templates were sequenced by the enzymatic method of SANGER, NICKLEN and COULSON (1977) as modified by CHEN and SEEBURG (1985) and ZAGURSKY *et al.* (1985).

RESULTS

Mutational spectra differ in otherwise isogenic *dam*⁺ and *dam*⁻ genetic backgrounds: The target we used to identify base pair changes induced in a *dam*⁻ strain is the 254-bp *mnt* repressor gene of phage P22 (SAUER *et al.* 1983). To permit the forward selection of mutations that inactivate the *mnt* gene, we used a fusion of the Mnt-repressible *ant* promoter to the *tetA* operon structural gene of plasmid pBR322. As diagrammed in Figure 1, plasmid pMQ151 is a derivative of pBR322 that contains a 500-bp fragment of P22 DNA substituted for the smaller *Eco*RI-*Hin*dIII region of pBR322. This fragment contains the *mnt* structural gene, and its promoter, as well as the Mnt-regulated *ant* promoter. Since the *tetA* gene is fused to the *ant* promoter, mutations that inactivate the *mnt* repressor gene or its operator permit the constitutive transcription of *tetA* from the *ant* promoter, enabling the plasmid to confer a tetracycline-resistant (Tet^R) phenotype.

TABLE 1

Constitutive mutations isolated in a *dam*⁻ host reveal a new target

Strain	Frequency ($\times 10^{-8}$)	Number	Locus	
			Operator	Gene
dam⁺	0.3	66	86%	14%
dam⁻	4.2	95	4%	96%

Mutant derivatives of plasmid pMQ151 that confer a tetracycline-resistant phenotype were isolated as described in MATERIALS AND METHODS. In a *dam*⁺ host, the majority of mutations map to the Mnt operator; in a *dam*⁻ host, the majority map to the *mnt* structural gene.

Table 1 shows that spontaneous Tet^R derivatives of *E. coli* carrying plasmid pMQ151 arise 14 times more frequently in a *dam*⁻ strain than in a *dam*⁺ background. These plasmid mutations were mapped to either the *mnt* structural gene or the Mnt operator (LUCCHESI, CARRAWAY and MARINUS 1986). The majority of mutations (86%) arising in a *dam*⁺ host were found to map to the Mnt operator. In contrast, most mutations (96%) arising in the *dam*⁻ background were found to map outside the Mnt operator, within *mnt*. (Since the critical determinants of Mnt operator function are only ten G:C base pairs (YOUDERIAN, MOYLE and SUSSKIND 1987), the spectra of operator-constitutive mutations are not so informative, and these mutations were not considered further in our analysis.) These results show that the spontaneous frequency of mutation to a Tet^R phenotype is elevated in a *dam*⁻ host. More important, the mutations arising in a *dam*⁻ host reveal a new target, the *mnt* structural gene.

The majority of *mnt*⁻ mutations isolated in a *dam*⁺ host is insertions and deletions: The positions and sequence changes of mutations mapping outside the Mnt operator isolated in the *dam*⁺ background are shown in Tables 2 and 3. Mutations due to the insertion of IS elements are the most frequent class (7 of

TABLE 2

Spectrum of mutations isolated in a *dam*⁺ host

Mutation	Number	*mnt*	P_{mnt}
IS insertions	7	5	2
Deletions	2	0	2
Total	9	5	4

All constitutive mutations isolated in a *dam*⁺ host are insertions or deletions. *Mnt* refers to the structural gene for repressor (base pairs +21 to +270); the promoter region P_{mnt} includes base pairs −35 to +17.

TABLE 3

Positions of mutations isolated in a *dam*⁺ host

Number	Position	Type
2	−34/−43	*del*
2	+16/+17	IS*1*R
1	+57/+58	IS*1*R
1	+59/+60	IS*1*R
1	+60/+61	IS*1*R
1	+71/+72	IS2L
1	+254/+255	IS*1*L

Base pairs are numbered with respect to the startpoint of *mnt* transcription (+1). The first position of the *mnt* translation initiation codon (ATG) is +21. IS*1*R and IS*1*L indicate IS*1* insertions for which the right and left ends, respectively, are closer to +1; *del* indicates a deletion. IS*1* and IS2 insertions were identified by comparison with their sequences defined by OHTSUBO and OHTSUBO (1978) and GHOSAL, SOMMER and SAEDLER (1979).

9), with IS*1* insertions recovered more frequently than IS2 insertions. Two of the insertions located between base pairs +16 and +17 are in the region of the 5′ untranslated leader mRNA sequence corresponding to *mnt*. Most of the insertions are in the promoter-proximal end of *mnt*, which includes amino acid residues of Mnt repressor critical for DNA binding (YOUDERIAN *et al.* 1983). The only other mutations that affect the *mnt* structural gene are deletions (2 of 9). These deletions remove base pairs −34 to −43, and extend from within the *mnt* promoter to within the Mnt operator.

The majority of *mnt*⁻ mutations isolated in a *dam*⁻ host is A:T→G:C transitions clustered at Dam sites: The majority of mutations mapping outside the Mnt operator isolated in the *dam*⁻ genetic background are transitions (73 of 91). Among these, A:T→G:C transitions are favored over G:C→A:T changes by a ratio of six to one. This distribution is seen in both the *mnt* promoter region, as well as in the structural gene for repressor (Table 4). This result may be anticipated for the promoter region, since it is very A:T-rich. The *mnt* structural gene, however, has approximately the same number of A:T and G:C base-pairs. Three hotspots for A:T→G:C transitions are evident at positions +41, +106 and +116 (Table 5). These are graphically shown in Figure 2. All three hotspots occur within six base pairs of the only two Dam recognition sites (5′-GATC-3′) located at base pairs +34 to +37 and +107 to +110, and are absent from the *dam*⁺ spectrum.

TABLE 4

Spectrum of mutations isolated in a *dam*⁻ host

Mutation	Number	*mnt*	P_{mnt}
A:T→G:C	62	55	7
G:C→A:T	11	10	1
A:T→T:A	1	0	1
A:T→C:G	2	2	0
G:C→T:A	1	1	0
IS insertions	6	6	0
Frameshifts	5	5	0
Deletions	3	0	3
Total	91	79	12

Mnt refers to the structural gene for repressor (base pairs +21 to +270); the promoter region P_{mnt} includes base pairs −35 to +17.

Insertion and deletion mutations were recovered less frequently from the *dam*⁻ background (4 of 91 and 5 of 91, respectively) than from the *dam*⁺ background. The number of mutations due to IS elements and deletions (9 of 91) is close to that expected from the background level of spontaneous mutation in the *dam*⁺ host. Since the frequency of mutation to a Tet^R phenotype is increased 14-fold in *dam*⁻ bacteria, these mutations may have arisen from events like those occurring in a *dam*⁺ host (Table 1). Most of the mutations in the *mnt* gene are clustered in its promoter-proximal end (Figure 2). This is not surprising, since this part of the gene is critical for DNA-binding (YOUDERIAN *et al.* 1983). Similar clusterings of missense mutations in the DNA-binding regions of Lac repressor (MILLER 1980) and phage lambda repressor (LIEB 1981) have been observed. We note that neither of these other targets contain Dam sites within their DNA binding regions, and that our choice of *mnt* as target may have been a fortunate one.

DISCUSSION

E. coli hosts carrying *dam*⁻ mutations exhibit higher rates of spontaneous mutation than otherwise isogenic *dam*⁺ hosts. We have shown that the spectrum of mutations isolated in a *dam*⁻ host in a small target, the Salmonella phage P22 *mnt* gene, differs markedly from that isolated in a *dam*⁺ host. Most of the mutations arising as a consequence of the *dam*⁻ defect are A:T→G:C transitions. Furthermore, we find three major hotspots for mutation that occur near Dam recognition (5′-GATC-3′) sites. Two are associated with the Dam recognition site at position +107 to +110. One is located a single base pair 5′ to this sequence, and one six base pairs after the site. The third hotspot occurs four base pairs before the Dam site at position +34 to +37.

TABLE 5

Positions of mutations isolated in a *dam*⁻ host

Number	Position	Codon change	Amino acid change
2	−35	T to C	
2	−34	T to C	
1	−33 to −45	*del*	
1	−31 to −48	*del*	
1	−31	G to A	
2	−11	A to G	
1	−11	A to T	
1	−7	T to C	
1	+7/+8	IS*1*R	
1	+8/+9	IS*1*R	
1	+23	ATG→AGG	fMet→Arg
2	+26	GCT→GTT	Ala1→Val
1	+28	AGA→GGA	Arg2→Gly
1	+35	GAT→GGY	Asp4→Gly
1	+36	−T	
1	+40	GAC→TAC	His6→Tyr
7	+41	GAC→CGC	His6→Arg
1	+44/+45	IS*1*R	
1	+45/+46	IS2L	
3	+46	AAC→GAC	Asn8→Asp
1	+47	AAC→AGC	Asn8→Ser
4	+52	CGT→TGT	Arg10→Cys
2	+70	AGG→GGG	Arg16→Gly
1	+71/+73	+G	
1	+80	TTA→TGA	Leu19→Ser
1	+81/+84	−A	
1	+89/+91	+G	
2	+92	GCG→GTG	Ala23→Val
11	+106	AGA→GGA	Arg28→Gly
1	+107	AGA→ATA	Arg28→Ile
2	+109	TCA→CCA	Ser29→Pro
2	+115	AAC→GAC	Asn31→Asp
1	+115	AAC→CAC	Asn31→His
19	+116	AAC→AGC	Asn31→Ser
1	+119	TCC→TTC	Ser32→Phe
1	+122	GAG→GGG	Glu33→Gly
1	+173/+174	IS*1*L	
1	+237/+238	IS*1*L	
4	+273	TGA→TGG	Stop→Trp

Base pairs are numbered with respect to the startpoint of *mnt* transcription (+1). The first position of the *mnt* translation initiation codon (ATG) is +21. IS*1*R and IS*1*L indicate IS*1* insertions for which the right and left ends, respectively, are closer to +1; *del* indicates a deletion. IS*1* and IS2 insertions were identified by comparison with their sequences defined by OHTSUBO and OHTSUBO (1978) and GHOSAL, SOMMER and SAEDLER (1979). "+" or "−" followed by a nucleotide base indicates a frameshift mutation.

GLICKMAN (1979) has shown that the elevated frequency of nonsense mutations in the *lacI* gene arising as a consequence of the *dam*⁻ defect are primarily G:C→A:T changes. In contrast, we find that the spectrum of *mnt*⁻ mutations obtained in a *dam*⁻ background is comprised of a majority of A:T→G:C transitions, precisely those substitutions that cannot be detected by the isolation of nonsense mutations. The second most frequent class of mutations is G:C→A:T changes. Frameshift mutations also comprise a significant fraction (6%) of the *dam*⁻ spectrum of *mnt*⁻ mutations. These results are consistent with the observation that these three classes of mutations revert at higher frequencies in a *dam*⁻ background (GLICKMAN 1979; MARINUS 1981). Both transitions and frameshifts appear to be subject to mismatch correction in *E. coli* and *Streptococcus pneumoniae* (CLAVERYS and LACKS 1985).

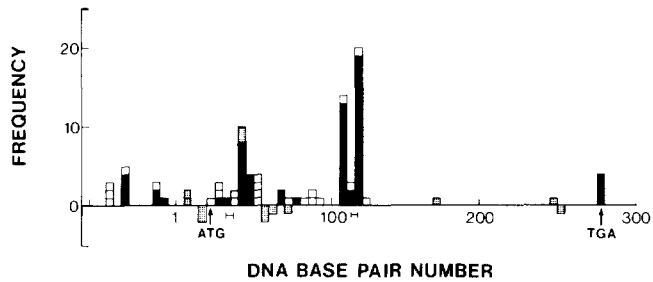

FIGURE 2.—Mutation spectra in *dam*⁺ and *dam*⁻ strains. Mutations above the line indicate those isolated in a *dam*⁻ strain, and those below the line in a *dam*⁺ strain. Stippled boxes indicate IS element insertions; black boxes indicate A:T→G:C transitions; and, white boxes indicate other mutational changes. Horizontal bars denote 5′ GATC 3′ sequences.

We assume that the spectrum of mutations in *mnt* that arise in a *dam*⁻ background reflects the spectrum of mispaired base pairs generated by replication that fail to be rectified by post-replicative mismatch repair. Therefore, we must conclude, like GLICKMAN (1979) and CLAVERYS and LACKS (1985), that a pyrimidine:purine mispair is the most frequent replicative error. These mistakes most likely result from the infidelity of DNA polymerase III, since the replication of the *mnt* gene on the plasmid we have used in this study is dependent on DNA polymerase III and not polymerase I (MINDEN and MARIANS 1985).

How, then, can we account for our surprising result that the majority of mutations obtained in the *dam*⁻ genetic background are clustered near Dam recognition sites? We propose that when methylated, the sequence 5′-GATC-3′ enables DNA polymerase III to replicate with greater fidelity. To test this hypothesis by genetic methods, we are constructing plasmids that code for Mnt repressors with wild-type amino acid sequence, but that lack 5′-GATC-3′ sequences, or have additional 5′-GATC-3′ sequences in the *mnt* gene. The absence or presence of hotspots associated with Dam sites will indicate whether this association is fortuitous. We also note that this hypothesis is open to simple, biochemical tests.

The role of 5′-GATC-3′ sequences in Dam-dependent post-replicative mismatch repair is to allow strand discrimination between parental and daughter strands. Consistent with this hypothesis, the MutH protein, required for mismatch repair, has weak endonuclease activity that cleaves 5′ to the guanine residue in 5′-GATC-3′ sequences (MODRICH 1987). This cleavage occurs on the unmethylated strand in a hemi-methylated duplex or on one of the two strands in an unmethylated duplex. Symmetrically methylated

duplexes are resistant to the action of the endonuclease.

These results would predict that there is increased MutH-dependent cleavage at 5′-GATC-3′ sequences in a *dam*⁻ mutant compared to wild type. Subsequent excision and resynthesis might introduce errors in DNA near Dam methylation sites. To test this hypothesis, we are currently determining the spectrum of *mnt*⁻ mutations in a *MutH* host.

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