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DNA methylation alters the pattern of spontaneous mutation in *Escherichia coli* cells (*mutD*) defective in DNA polymerase III proofreading

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Summary

We have shown previously that *dam* mutants of *Escherichia coli* have a weak mutator phenotype which generates mostly transition mutations in the P22 *mnt* gene. In contrast, in *mutD5* cells, which have a strong mutator phenotype, transversion mutations were the most prevalent. A *dam-16 mutD5* strain, defective in both DNA polymerase III associated-proofreading and Dam-directed mismatch repair, exhibits a strong mutator phenotype but, surprisingly, its mutation spectrum is similar to that of the *dam* rather than the *mutD* parent. The most likely explanation is that Dam-directed mismatch repair in the *mutD5* strain corrects most of the potential transition mutations (therefore yielding transversions) in the newly synthesised strand. When the *dam-16* allele is present together with *mutD5* a reduced efficiency of repair as well as loss of strand discrimination and misdirected repair results in the appearance of transition mutations at high frequency.

Escherichia coli mutant cells which do not have Dam (DNA adenine methylase) methylation show a variety of phenotypic traits compared to wild-type (Marinus, 1987; Barras and Marinus, 1989). Among these is an increased spontaneous mutation rate. This results from the inability of the Dam-

directed repair system (Meselson, 1988; Modrich, 1989) to discriminate correctly between parental and newly synthesized DNA strands. In the wild-type, Dam-directed repair preferentially removes replication errors in the newly-synthesized (under-methylated) strand rather than the parental (fully methylated) strand. In the absence of Dam methylation, this directionality is lost and repair can occur on either strand. Repair of the parental strand, therefore, leads to an increased mutation rate.

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of single-base substitutions in various experimental systems but the spectrum can be modified by cultural conditions (Fowler et al., 1974, 1986; Isbell and Fowler, 1989; Piechocki et al., 1986; Schaaper, 1988). The *mutD* gene encodes the ϵ -subunit of DNA polymerase III holoenzyme and this subunit has 3' → 5' exonuclease (proofreading) activity (DiFrancesco et al., 1984; Scheuermann et al., 1984). In association with the α -subunit, ϵ removes mismatched bases at the end of the growing polynucleotide chain (Maki and Kornberg, 1987). An increased mutation rate would, therefore, be expected from a reduction in proofreading activity.

In order to determine the specificity of repair by the two repair systems mentioned above, we have defined the mutation spectra in *dam* and *mutD5* strains by DNA sequence analysis of the *mnt* gene as a target (Carraway et al., 1987; Wu et al., 1990). Transition mutations predominated in the *dam* strain and, as expected, almost identical patterns were also found in *mutH* and *L* bacteria. In contrast, transversion mutations were the major class recovered from *mutD5* cells. A majority of these occurred at thymine-109 replacing it with either adenine or guanine. We were interested to then find out if the mutation spectrum in a *dam mutD* double mutant would be the same as either of the parent strains or different.

Materials and methods

Bacterial strains, plasmids and phages

The strains, plasmids and phages used are described in Table 1. All strains were grown in Brain Heart Infusion broth (BH broth) unless otherwise described. The minimal medium used was that of Davis and Mingioli (1950).

Construction of dam mutD double mutant

A *dam-16 mutD5* derivative of AB1157 was constructed by the mobilization of the *dam-16* allele (a deletion mutation with a kanamycin resistance substitution; Parker and Marinus, 1988) from GM2807 by conjugation into the *mutD5* strain, ES1578. Recombinants were selected on kanamycin

TABLE 1
BACTERIAL STRAINS, PHAGES AND PLASMIDS

<i>E. coli</i> strains	Characteristics	Source or Ref.
AB1157	<i>thr-1 ara-1 leuB6</i> Del(<i>gpt-proA</i>)62 <i>lacY1 tsx-33 supE44 galK2 hisG4(Oc) rfb-1 mgl-51 rpsL31 kdgK51 xyl-5 mtl-1 argE3 thi-1</i>	E. Adelberg
AB1874	<i>lac-19/F' lac</i> (F-42)	Laboratory stocks
ES1578	AB1157 but <i>mutD5 proA</i> ⁺	E. Siegel
GM379	GM4708/F' <i>lac</i> (F-42)	This study
GM2807	<i>thi-1 relA1 dam-16*</i> Hfr (PO68)	Laboratory stocks
GM3819	AB1157 but <i>dam-16</i>	Parker and Marinus (1988)
GM4708	ES1578 but <i>dam-16</i>	This study
GM5018	GM379/pPY97	This study
KL14	<i>thi-1 relA1</i> Hfr (PO68)	K.B. Low
MM294	<i>endA1 thi-1 hsdR17 supE44</i>	Meselson and Yuan (1968)
<i>Bacteriophages</i>		
vir	virulent phage	Laboratory stocks
<i>immP22hy1</i>	<i>immC</i> ^{P22}	N. Yamamoto
<i>immP22dis</i>	<i>immI</i> ^{P22} <i>immC</i> ^{P22}	N. Yamamoto
<i>sfiA::lacZ</i>	<i>sfiA::lacZ cIind</i> ⁻	Huisman and D'Ari (1983)
P1	generalised transducing phage	Laboratory stocks
<i>Plasmids</i>		
pMQ191	pACYC184 replicon, <i>tet</i> , <i>P_{tac-dam}</i>	Laboratory stocks
pPF3	pBR322 replicon, <i>bla</i> , <i>P_{tacUV5-}</i> <i>dnaQ</i>	P. Foster
pPY97	pBR322/M13 replicon, <i>bla</i> , <i>mnt</i> , <i>P_{ant-tet}</i>	Rewinski and Marinus (1987)

* *dam-16* is a substitution (insertion/deletion) mutation in which the *dam* gene has been replaced with a 1.1-kb fragment conferring kanamycin resistance (Modrich, 1989).

cin and streptomycin containing medium and initially screened for sensitivity to 2-aminopurine [2AP] (indicative of *dam*) and a high level of mutability on medium containing 100 $\mu\text{g}/\text{ml}$ rifampicin (indicative of *mutD*). Colonies resistant to both kanamycin and streptomycin occurred at approximately 10^{-3} per donor cell. The *mutD5* marker of GM4708 could be transferred out of this strain into AB1157 using P1 transduction and selecting for the closely linked ProA^+ marker.

An F-*lac* (F-42) derivative of GM4708, GM379, was obtained by conjugation with AB1874 and this strain was transformed with pPY97 to yield GM5018. An F-*lac* derivative was used in all previous studies to allow single-strand plasmid isolation after infection with single-stranded DNA phages.

Plasmid DNA isolated from GM5018 when assayed for digestion with *DpnI*, *MboI* and *Sau3AI* was sensitive to *MboI* and *Sau3AI* digestion and resistant to *DpnI* digestion confirming that GM4708 is Dam^- . Resistance to *DpnI* and sensitivity to *MboI* digestion are indicative of DNA lacking adenine methylation at GATC sites,

TABLE 2
COMPARISON OF MUTABILITY OF WILD-TYPE, *dam-16*, *mutD5* AND *dam-16 mutD5* STRAINS

Strain	Resistant colonies/ 10^8 cells plated		
	Nalidixic acid	Rifampicin	Mnt
AB1157 (wild-type)	7 ± 2	6 ± 5	1 ± 1
ES1578 (<i>mutD5</i>)	3900 ± 330	1700 ± 210	1500 ± 510
GM3819 (<i>dam-16</i>)	40 ± 4	10 ± 1	5 ± 4
GM4708 (<i>dam-16 mutD5</i>)	6000 ± 63	3200 ± 150	9000 ± 6600
GM4708/pPF3	9 ± 2	50 ± 9	NT ^a
GM4708/pPF3/pMQ191	0.2	18 ± 7	NT

For resistance to nalidixic acid and rifampicin, 10^8 stationary phase cells were plated on BH medium containing the antibiotics at 100 $\mu\text{g}/\text{ml}$ and incubated overnight at 37°C prior to counting resistant colonies. Mutations in the *mnt* region were scored as those resistant to 40 $\mu\text{g}/\text{ml}$ ampicillin and 3.5 $\mu\text{g}/\text{ml}$ tetracycline using pPY97/F-42 derivatives. Plasmids pPF3 and pMQ191 encode the wild type *mutD* and *dam* genes respectively.

^a NT, not tested.

Sau3AI cuts at these sites regardless of the methylation state.

Measurement of spontaneous mutation frequency

The spontaneous mutation frequency of various strains was measured by spreading 10^8 stationary phase cells (or a smaller number if necessary) grown in BH broth onto BH plates containing 100 $\mu\text{g}/\text{ml}$ nalidixic acid or rifampicin. Resistant colonies were counted after overnight incubation at 37°C.

β -Galactosidase assays

Strains to be assayed were grown to a culture density of 30 Klett units with shaking at 37°C after a 10^{-2} dilution of an overnight culture. The cultures were divided and mitomycin C (MC) was added to one half to a final concentration of 0.25 $\mu\text{g}/\text{ml}$ and incubation continued for an additional 90 min before sampling. β -Galactosidase activity was assayed as described by Miller (1972).

Isolation and characterization of *mnt* mutants

The procedures for isolating, identifying and DNA sequencing of *mnt* mutations have been fully described (Rewinski and Marinus, 1987). Briefly, tetracycline resistant derivatives of GM5018 were isolated by plating 2×10^8 stationary phase cells on BH plates containing 40 $\mu\text{g}/\text{ml}$ ampicillin and 3.5 $\mu\text{g}/\text{ml}$ tetracycline. Strains containing mutations in the Mnt operator were detected by testing for sensitivity of lambda *immP22hy1* lysogens to lambda *vir* and immunity to lambda *immP22hy1* and lambda *immP22dis* phages and in some instances DNA sequencing of the *mnt* region of the purified mutant pPY97 plasmids. Plasmid DNA was isolated (Holmes and Quigley, 1981) from *mnt* isolates and used to transform strain MM294. Plasmid DNA from clonally purified transformants was isolated and the *mnt* region sequenced (Rewinski and Marinus, 1987).

Results

Properties of the double mutant

The spontaneous mutation frequencies to rifam-

picin and nalidixic acid resistance of isogenic strains bearing *dam-16* (GM3819), *mutD5* (ES1578) or both mutations and the wild-type (AB1157) are presented in Table 2. The frequency of mutation in *mnt* in these strains is also presented in Table 2. This shows that GM4708 has a spontaneous mutation frequency of similar magnitude to that of ES1578. Transformation of Gm4708 with first pPF3 (encoding the wild-type *mutD* [*dnaQ*] gene) and then a compatible *dam*⁺ plasmid, pMQ191, reduced the strain's mutability to a low level (Table 2). The results in Table 2 also show that introduction of a wild-type *mutD* gene on a high copy plasmid reverses the dominant phenotype of a *mutD5* allele (Degnan and Cox, 1974).

Strain GM379 is an F-*lac* (F-42) derivative of GM4708 and strain GM5018 is GM4708 transformed with pPY97. All the *dam-16 mutD5* strains (GM4708, GM379 and GM5018) grew slowly. GM4708 showed an extended lag phase and slower exponential phase growth when compared with ES1578 (*mutD5*), GM2807 (*dam-16*), GM3819 (*dam-16*) and AB1157 (wild-type) (Fig. 1). Stationary phase cultures of GM4708 have a reduced number of colony-forming units (cfu) when compared to the other strains, having approximately

1×10^9 cfu/ml of stationary-phase culture compared to approximately 3×10^9 cfu/ml for the other strains.

To ascertain whether this result might be due to an increased level of filamentation by GM4708, cells from fresh overnight colonies of strains AB1157, GM2807, GM3819, ES1578 and GM4708 were observed by light microscopy. All 4 mutant strains showed a higher frequency of filamentous cells when compared to AB1157. However this was particularly pronounced in GM4708 (approximately 4% or more of cells were filamentous compared to less than 1% for the other strains examined) with filaments often longer than 20 μ m long.

In an effort to determine if this filamentation was a result of induction of SOS genes, AB1157, ES1578, GM3819 and GM4708 were made lysogenic for a phage lambda derivative carrying a *sfiA::lacZ* gene fusion (Huisman and D'Ari, 1983) and assayed for β -galactosidase levels. Table 3 shows that, in the absence of MC, both *dam-16* strains (GM3819, GM4708) contain a higher level of enzyme than the wild-type or *mutD5*. Growth in the presence of 0.25 μ g/ml mitomycin C resulted in high β -galactosidase levels in all strains (Table 3). These results suggest that the higher basal level of the SOS regulon is a major contributing factor to filamentation, slow growth and poor viability of the double mutant.

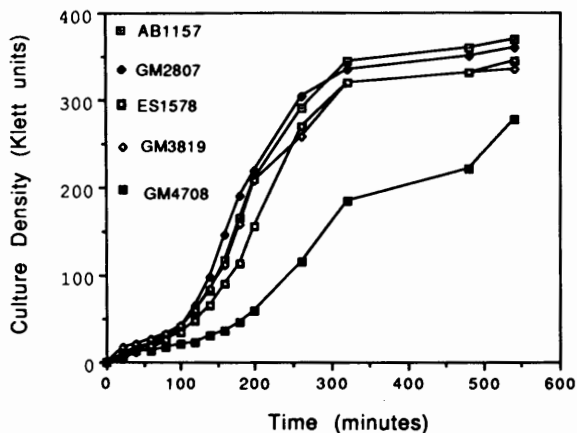


Fig. 1. Comparison of growth rates of wild-type, *dam*, *mutD* and *dam mutD* strains. An inoculum of 10^8 stationary phase cells of each strain was added to 10 ml Brain Heart Infusion broth and incubated with shaking at 37°C.

TABLE 3

β -GALACTOSIDASE LEVELS IN VARIOUS STRAINS LYSOGENIC FOR LAMBDA *sfi::lacZ* IN THE PRESENCE OR ABSENCE OF MITOMYCIN C (MC)

Lysogenic derivative of strain	Units of β -galactosidase	
	- MC	+ MC
AB1157 (wild)	58	1008
ES1578 (<i>mutD5</i>)	126	2722
GM3819 (<i>dam-16</i>)	296	2354
GM4707 (<i>mutD5 dam-16</i>)	340	1847

β -Galactosidase was measured as described by Miller (1972). Non-lysogenic strains contained 1–2 units of enzyme. MC was added to a final concentration of 0.25 μ g/ml and the culture incubated for a further 90 min before sampling.

TABLE 4
POSITION OF MUTATIONS IN THE *mnt* GENE
ISOLATED FROM A *dam-16 mutD5* STRAIN

Number	Position	Codon change	Amino acid substitution
1	- 34/ - 44	deletion	Promoter
1	- 33	G to A	Promoter
1	+ 40	CAC to TAC	His6 to Tyr
2	+ 41	CAC to CGC	His6 to Arg
1	+ 46	AAC to GAC	Asn8 to Asp
1	+ 52	CGT to TGT	Arg10 to Cys
1	+ 89	AGG to AAG	Arg22 to Lys ^a
1	+ 89/ + 91	+ G	Frameshift
1	+ 106	AGA to TGA	Arg28 to Ter (Opal) ^a
3	+ 106	AGA to GGA	Arg28 to Gly
4	+ 116	AAC to AGC	Asn31 to Ser
1	+ 116	AAC to ATC	Asn31 to Ile ^a
1	+ 125	TTG to TCG	Leu34 to Ser ^a
1	+ 195/ + 196	+ A	Frameshift ^a

Nucleotides are numbered with respect to the transcription start site. The start codon is at positions 21-23 and the termination codon at positions 271-273. Mutations were isolated and characterised as described in Materials and Methods. The asterisk denotes mutations not seen previously in other strain backgrounds.

Isolation and characterization of mnt mutants in a dam-16 mutD5 background. The experimental system has been described fully previously (Carraway et al., 1987; Wu et al., 1990). In summary, pPY97, a pBR322 derivative, contains a region of bacteriophage P22 including the *mnt* (maintenance of lysogeny) repressor gene and its operator fused to the *tet* gene. This places the *tet* gene under the negative control of Mnt repressor which when bound to its operator prevents transcription from P_{ant}, a promoter which overlaps the Mnt operator. Cells containing wild-type Mnt are tetracycline-sensitive whereas mutants in *mnt* or its operator result in a tetracycline-resistant phenotype.

Presumptive *mnt* mutants of GM5018 were isolated as tetracycline-resistant colonies on BH plates containing 40 µg ampicillin/ml and 3.5 µg

tetracycline/ml. These isolates retained the characteristics of GM4708 (i.e., sensitivity to 2AP and high mutability and the *mutD5* marker could still be transduced out of these isolates).

44 tetracycline-resistant colonies were chosen for further study. Of these 24 (55%) were mutations in the Mnt operator. The remaining 20 isolates contained mutations within the *mnt* gene. The spectrum of mutations obtained is shown in Table 4. The mutation spectrum of the *dam-16 mutD5* strain background is compared to that obtained from a *dam-3* strain [data from Carraway et al. (1987)] and a *mutD5* strain (ES1578) [data from Wu et al. (1990)] in Fig. 2. Table 5 gives a summary of the type and proportion of mutations observed in all strain backgrounds studied using this system to date. Unexpectedly the spectrum of the double mutant resembles that of the *dam* mutant, rather than that of the *mutD5* strain as might have been expected. The mutations isolated in the *dam-16 mutD5* were predominantly transitions (75%), with hotspots at base pairs + 41, + 106 and + 116 in the *mnt* gene. 55% of all mutations occurred at these 3 sites. These were features of spectra obtained in *dam*, *mutH* and *mutL* backgrounds (Carraway et al., 1987, 1988; Rewinski and Marinus, 1987; Wu et al., 1990). The small sample size (20 mutations compared to up to 92 in other surveys, ref. 2) was thought justified as the spectrum is so similar to that of *dam*. Using a two sample Wilcoxon test the *dam* and *dam mutD* spectra could not be distinguished.

Discussion

The slow growth rate, reduced viability of stationary-phase cultures, increased filamentation and a sub-induced basal level of *sfiA* expression are consistent with poor viability of the double mutant. These phenotypic traits are present in *dam* mutants to a lesser degree and are thought to be due, in part, to the presence of breaks in DNA (Marinus, 1987; Barras and Marinus, 1989). Associated with these traits is the inviability of *dam* mutant alleles in combination with mutant alleles of, for example, *recA* and *recBCD*.

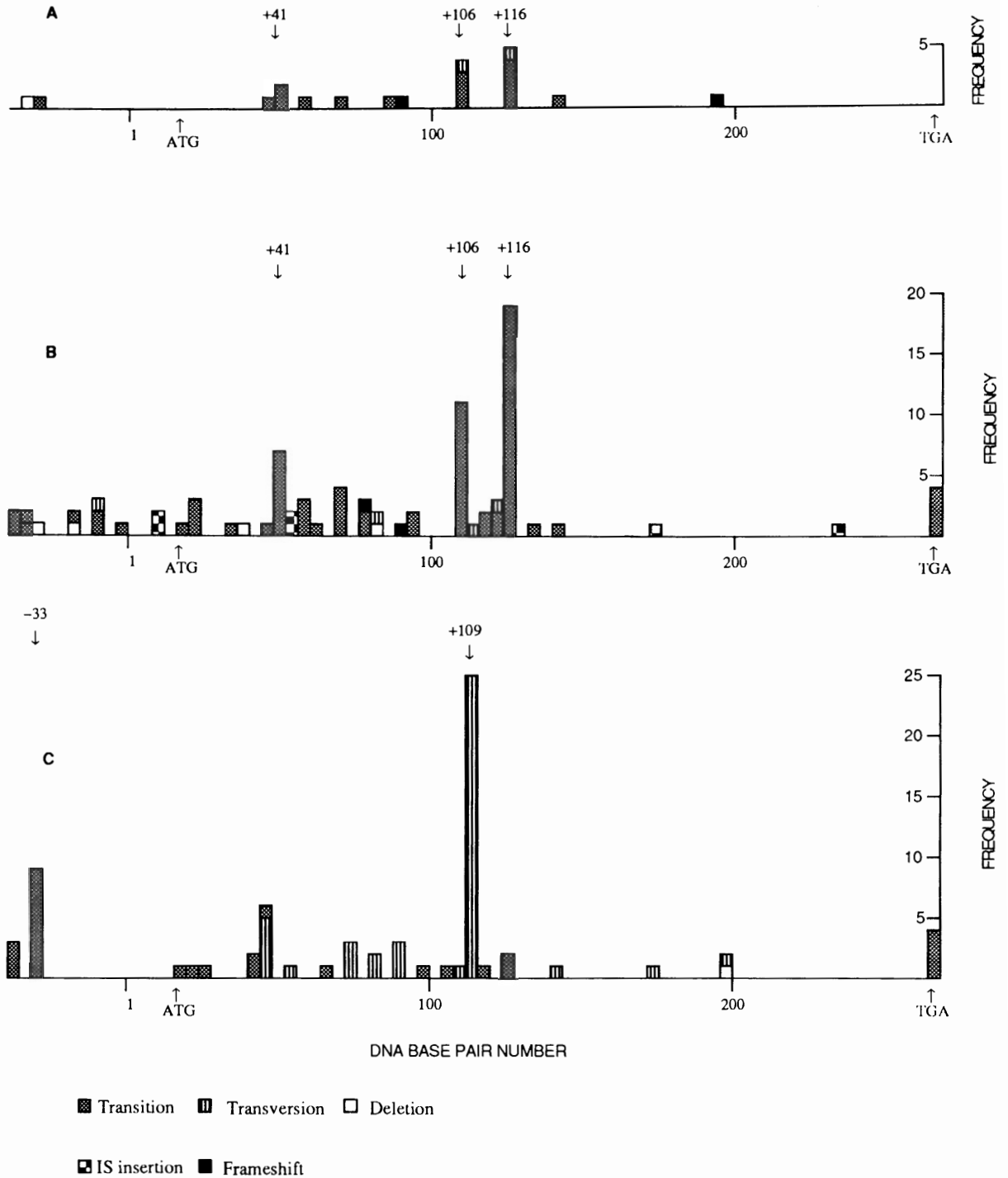


Fig. 2. Distribution of mutations in the *mnt* gene obtained from *dam*, *mutD* and *dam mutD* strains. Mutation spectra are shown for (A) *dam-16 mutD5*, (B) *dam-3*, (C) *mutD5* backgrounds. The data for the *dam-3* and *mutD5* strains are taken from Carraway et al. (1987) and Wu et al. (1990) respectively. Frequency indicates the number of mutational occurrences at a particular site for each strain. Hence, comparisons between strains cannot be made.

Strain GM5018 was isolated as a recombinant from a cross as described in Materials and methods. The size of the double mutant recombinant class in this cross was lower than expected suggesting that the combination of *dam* and *mutD5* may be inviable and that GM5018 may be able to grow because of a second-site suppressor mutation. If GM5018 does indeed carry an additional suppressor mutation, it is important to note that it has no effect on spontaneous mutability since complementation with the wild-type alleles of *dam* and *mutD* reduce mutability of GM5018 to background levels (Table 2).

The *mutD* gene has been shown to be inducible under conditions of mutagenic stress (Quiñones et al., 1988) and there appears to be an interaction between proofreading and the SOS regulon (Foster and Sullivan, 1988; Piechocki et al., 1988). It is unlikely that SOS-induced mutations are being

produced at high frequency in the double mutant since these are typically transversions. We do not know if there is increased expression of the *mutD5* gene in GM4708 or what the consequences of such overproduction might be.

It might have been expected that the strong mutator phenotype of the *mutD5* allele may have exerted a degree of dominance over the relatively weak *dam* mutator phenotype with regard to the spectrum of mutations observed (Table 2). However, this does not appear to be the case and the most likely explanation for our results is that proposed by Schaaper (1989) and Schaaper and Radman (1989). It was observed that *mutD* strains were deficient in mismatch repair because the high mutagenic load in rapidly growing *mutD5* cells leads to saturation of mismatch-repair capability. Under these conditions a mutation spectrum similar to that of mismatch repair-deficient cells was found whereas a slow growing *mutD5* strain has a different spectrum because mismatch repair is not saturated.

Since Dam-directed repair is more effective at correcting transition than transversion mispairs, we assume that in the *mnt* system in *mutD5* cells, Dam-directed mismatch repair is fully operational and removes most potential transition mutations but not the *mutD5* induced transversions. In the double mutant, the *mutD* transversions are still present but are now overwhelmed by transition mutations. We speculate that the Dam-directed mismatch repair system is saturated and therefore results in a spectrum typical of mismatch-repair deficient cells. In addition, residual Dam-directed repair in the absence of Dam methylation would result in misrepair of the parental strand half of the time yielding an increased mutation frequency.

A corollary of the above is that the mutation spectrum in *mutD5* strains also mutant at the *mutH*, *L* or *S* genes should be that of the mismatch repair defective strains. This is indeed the case (cited in Schaaper, 1989).

If our speculation is correct, then mismatch repair in the double mutant should be less efficient than in the *mutD5* strain. We are currently testing this hypothesis using heteroduplexes containing

TABLE 5

TYPES AND PROPORTION OF MUTATIONS IN THE *mnt* GENE FROM WILD-TYPE AND MUTATOR STRAINS

Type of mutation	Allele					
	Wild-type	<i>dam-3</i>	<i>mutH34</i>	<i>mutL25</i>	<i>mutD5</i>	<i>dam-16 mutD5</i>
IS element	77	7	2	0	0	0
Deletion	23	3	2	2	1	5
Frameshift	0	5	0	7	0	10
Transition	0	80	96	87	37	75
AT to GC	0	68	68	67	16	55
GC to AT	0	12	28	20	21	20
Trans-						
version	0	4	0	5	62	10
AT to TA	0	1	0	0	17	10
AT to CG	0	2	0	2	36	0
GC to TA	0	1	0	0	0	0
GC to CG	0	0	0	3	9	0

The numbers in the Table indicate the percent total mutations for each strain. Data for the *dam-3* strains are from Carraway et al. (1987); for *mutH* from Rewinski and Marinus (1987); and for *mutD* and *mutL* from Wu et al. (1990). The figures in the Table indicate the proportion of mutations in a particular class as a percentage of the total.

mismatched bases. We will also test the efficiency of repair in *dam* mutants to determine if the increased mutation rate is due only to misdirected repair and not a concomitant reduction of repair capability.

It has been hypothesized that the very prominent transversion hotspot in *mnt* at position +109 observed in the *mutD5* background was due to the methylation of the adjacent adenine at +108 by the Dam methylase (Wu et al., 1990). This study was, in part, an attempt to test the validity of this hypothesis. The radically different spectrum obtained from GM5018 does not allow a conclusion in support of or against this idea to be drawn. This hypothesis is being further investigated by the site-directed mutation of the *mnt* gene and the study of hotspot mutations.

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