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The isolation and characterization of the *Escherichia coli* DNA adenine methylase *(dam)* gene

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ABSTRACT

The E. coli dam (DNA adenine methylase) enzyme is known to methylate the sequence GATC. A general method for cloning sequence-specific DNA methylase genes was used to isolate the dam gene on a 1.14 kb fragment, inserted in the plasmid vector pBR322. Subsequent restriction mapping and subcloning experiments established a set of approximate boundaries of the gene. The nucleotide sequence of the dam gene was determined, and analysis of that sequence revealed a unique open reading frame which corresponded in length to that necessary to code for a protein the size of dam. Amino acid composition derived from this sequence corresponds closely to the amino acid composition of the purified dam protein. Enzymatic and DNA:DNA hybridization methods were used to investigate the possible presence of dam genes in a variety of prokaryotic organisms.

INTRODUCTION

The E. coli dam (DNA adenine methylase) gene codes for an enzyme which methylates within the sequence GATC (1-3). When DNA has been modified by the dam methylase, it is no longer susceptible to cleavage by the restriction endonuclease MboI (2,4).

The dam methylase is not part of a restriction modification system, but rather has been thought to act in post-replication mismatch repair. There are several lines of evidence for its involvement in mismatch repair. First, in heteroduplex lambda phage DNA having only one methylated strand, the repair system will usually correct the unmethylated strand to match the methylated strand; fully methylated mismatched heteroduplexes are not corrected (5,6). Second, E. coli strains in which the dam methylase is either not produced (dam^-) or overproduced (dam^s) are hypermutable (7-10). Third, the combination of the dam3 mutant allele, which has no detectable dam activity, with mutants in DNA repair functions such as polA, lexA, recA, recB or recC is lethal (11).

Furthermore, the E. coli dam protein may play a role in DNA replication: the sequence methylated by the dam enzyme, GATC, occurs at a very high

frequency (11 times within 245 base pairs) at the E. coli origin of replication (12,13). It also tends to occur with high frequency near or at the ends of Okazaki fragments (14,15).

In addition to functional considerations, the E. coli dam enzyme is noteworthy because it shares sequence specificity with a number of Type II restriction endonucleases and methylases (16). In particular, we were interested in determining whether the dam gene has any sequence homology to genes encoding Type II restriction methylases with the same specificity. Therefore, we undertook the isolation and characterization of the dam methylase gene from E. coli.

MATERIALS AND METHODS

(a) Bacterial strains.

The E. coli strains used were GM119 (dam3; Marinus, unpublished observation); SK1036 (dam4) (10) and HB101, (dam^+), (17). The origin of the bacterial species found in Table 3 is given in Roberts (16), except for the following species: E. aerogenes, E. carotovora and R. meliloti came from J. Zyskind; S. typhimurium came from S. Schlagman.

(b) DNA preparation.

The E. coli plasmids used in cloning and sequencing were isolated by the cleared lysate method (18), followed by banding on CsCl gradients containing ethidium bromide. ''Mini''preparations of plasmid DNA were prepared by the procedure of Klein et al. (19). Chromosomal DNA preparations from the various bacteria were made by the method of Marmur (20).

(c) Restriction enzymes and DNA end-labeling.

The enzymes Sau3AI, BclI, RsaI, and TaqI were prepared in this laboratory by P.A. Myers. DdeI was a gift from R. Meagher. Digests with these enzymes were carried out in buffer containing 6 mM Tris-HCl pH 7.9, 6 mM $MgCl_2$, and 6 mM $SHCH_2CH_2OH$. The enzymes BamHI, PvuI, PvuII, MboI, HindIII, and SalI were purchased from New England Biolabs; HpaI was purchased from Bethesda Research Labs. For these enzymes, we used the buffers recommended by their respective manufacturers.

Calf alkaline phosphatase was obtained from Boehringer-Mannheim, and purified further by passage over a DEAE-cellulose column. It was used as described previously (21). T4 polynucleotide kinase (Boehringer-Mannheim), was used as described by Chaconas and van de Sande (22). The 5'[γ^{32}P] ATP (3000 Ci/mmol) was purchased from Amersham, Inc.

(d) Determination and analysis of base sequence.

The chemical method of Maxam and Gilbert (23) was used to determine the base sequence of the cloned insert in pdam118. The specific reactions used were dimethylsulfate (G), formic acid (G + A), hydrazine (T + C), and hydrazine plus NaCl (C). Reaction products were resolved on 0.2 mm thick 6, 8, or 12% polyacrylamide gels containing 8 M urea and a Tris-borate buffer (24). Gels were autoradiographed using Kodak XR-1 X-Ray Film, sometimes with the aid of DuPont Cronex ''Lightning Plus'' intensifying screens.

The resulting data were assembled and analysed using computer programs which are described elsewhere (25-27). Further analysis involved the use of several programs in the MOLGEN-SEQ collection on the SUMEX computer system at Stanford University (28).

(e) Cloning dam in E. coli.

pGG503, containing the dam function on a 23 kb insert into pBR322 (10,29) was our starting material. 10 µg of plasmid was subjected to partial cleavage with Sau3A to give fragments averaging 2-5 kb in length. 1.0 µg of the digested plasmid was ligated to phosphatase-treated pBR322 (0.1 µg) using T4 DNA ligase (N.E. Biolabs) under conditions suggested by the manufacturer. The ligation reaction was used to transform E. coli SK1036 (dam$^-$) cells by the $CaCl_2$-heat shock method (30). After 1 hour, 5 ml LB broth was added to the transformation mix and the cells grown for 2 hours in the presence of ampicillin (100 µg/ml). At that time the cells were harvested, resuspended in 5 ml LB with ampicillin and grown overnight. Plasmids were isolated from the culture by the method of Klein et al. (19). 2 µg of the recombinant plasmids were extensively cleaved by incubation with 10 units of MboI for 3 hours at 37°C. The reaction was terminated by heating to 68°C for 5 minutes and then used to transform E. coli SK1036 cells. Transformants were selected on LB plates containing 100 µg/ml ampicillin, and screened by replica plating for sensitivity to 2-aminopurine in LB plates (400 µg/ml; Vega Biochemicals) (2-aminopurine is an adenine analog that is lethal to dam$^-$ cells at high concentrations (8)). Colonies that could grow in the presence of 2-aminopurine were picked from control plates, their plasmids isolated and challenged by MboI. Those plasmids not cleavable by MboI were further analyzed to determine the size of the insert.

(f) Presence of dam in other organisms.

To test for methylation of the GATC sequences in other organisms, 2 µg of bacterial DNA were digested with 3 units of MboI enzyme and the products resolved by electrophoresis on a 1% agarose gel (31). To test for sequence

homology, 2 μg of each DNA were digested with 3 units of HindIII enzyme and the digests run out on 1% agarose gels. These DNAs were then transferred to nitrocellulose filters (Schleicher and Schuell, BA85) by the Southern method (32). Hybridization probes (pBR322, Adenovirus-2 DNA, and the isolated BamI-PvuII fragment from pdam118) were labeled by nick-translation as described (33). Hybridization and filter washes were performed at 42°C as described by Bukhari et al. (34) with the modifications of Chaconas et al. (35). Hybridizations were performed under less stringent conditions in which the filters were first incubated at 15°C and washed extensively at 4°C.

RESULTS

(a) Localization of the dam gene.

Details of the cloning procedure are given in the Methods section and diagrammed in Fig. 1. Prior to the second transformation step, only 1 in 10^5 plasmids escapes MboI restriction; of the secondary transformants, 80% possess a Dam$^+$ phenotype (i.e., their isolated plasmids are resistant to MboI cleavage). The smallest of the Dam$^+$ plasmids, designated pdam117, contains a 1.6 kb insert. pdam117 was further analyzed to determine the position of the dam gene within the 1.6 kb insert. This was first done by a series of subcloning experiments based upon extensive restriction site analysis of the plasmid.

The results of these subcloning experiments are illustrated in Figure 2. Cleavage of pdam117 with PvuII followed by religation led to a clone (pdam118) which was phenotypically Dam$^+$, but carried an insert which was approximately 400 base pairs shorter than that of pdam117 (i.e., 1.14 kb). Two other subclones were constructed by splitting the pdam117 insert at its internal BamHI site. The 500 base pair ''Bam-Bam'' fragment was inserted into the BamHI site of pBR322, while the remainder of pdam117 (minus the Bam-Bam piece) was recircularized by ligation. Both plasmids, when used to transform GM119 cells were found to be Dam$^-$. These clones were designated as pxdam1 and pxdam2, respectively. Finally, the plasmid pdam118 was cleaved with PvuII and HpaI, the resulting large fragment recircularized, and transformed into GM119 cells. This construct, designated pxdam3, also resulted in cells which were Dam$^-$. These experiments suggest that the dam gene is situated with one end between the two BamHI sites and the other between the HpaI and PvuII sites within the pdam118 insert (Figure 2).

(b) Nucleotide sequence of the dam gene.

The strategy used in sequencing pdam118 by the Maxam-Gilbert method is

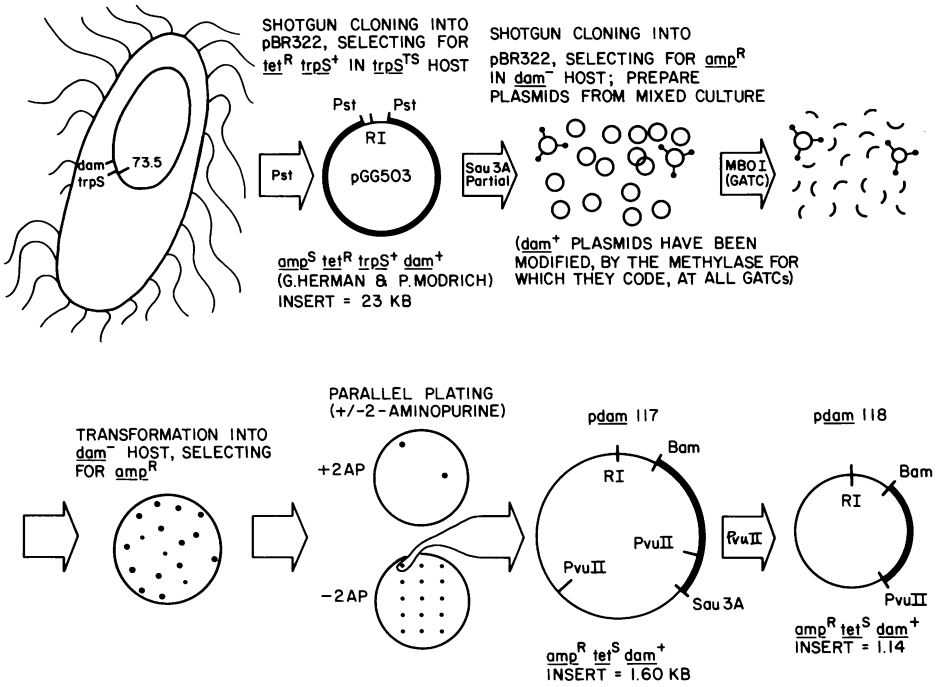

Figure 1: Cloning strategy for the *E. coli* *dam* methylase gene. A clone bank of the *E. coli* genome was constructed by Clarke and Carbon (50). This bank was screened by Modrich and Herman (29) for clones containing the *trpS* gene, since *trpS* had previously been genetically mapped proximal to the *dam* locus (51,52). A 23 kb *Pst* fragment, containing both the *dam* and *trpS* functions was inserted into pBR322 and the construct designated pGG503 (29).

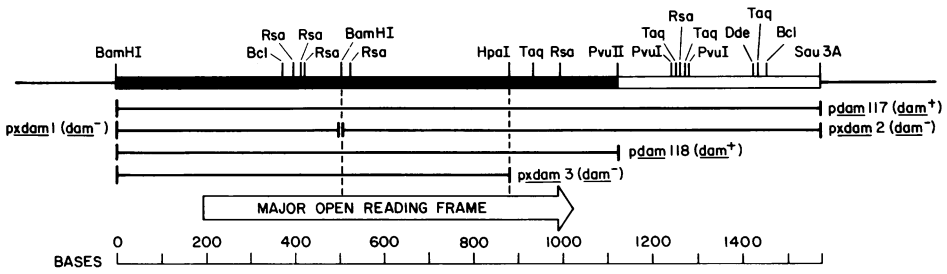

Figure 2: Restriction map of the insert from pdam117 and derivative plasmids. The position and orientation of the *dam* methylase gene is indicated.

shown in Figure 3. The completed sequence (Figure 4) was analyzed by computer for the presence of an open reading frame which could accommodate a product the size of the dam methylase. The longest available reading frame is 834 nucleotides in length, beginning with an AUG codon at position 195 of the insert, and ending with a UAA terminator at nucleotide 1029. The dam methylase has been purified to homogeneity (29) and the amino acid composition of the dam protein determined (P. Modrich, pers. comm.). We have compared the actual amino acid composition to the predicted composition of the putative protein from the 834 base open reading frame (Table 1): the comparison shows an excellent agreement in both size and composition between the actual and putative proteins.

(c) The presence of dam genes in other organisms.

A variety of prokaryotic organisms were tested for both the presence of a ''dam-like'' function and sequence homology to the cloned dam gene. To evaluate whether an organism has a dam methylase, we digested its DNA with MboI; if methylated in vivo, the GATC sequences are completely resistant to MboI cleavage. To determine whether an organism has DNA sequences homologous to the dam gene, we used Southern blot hybridization analysis. The results of both types of experiments are summarized in Table 2. It is clear that there are DNA sequences homologous to dam in all the Enterobacteriaceae and Haemophilus species tested; the DNA of all these bacteria are also protected against MboI cleavage. It is therefore probable that these organisms all

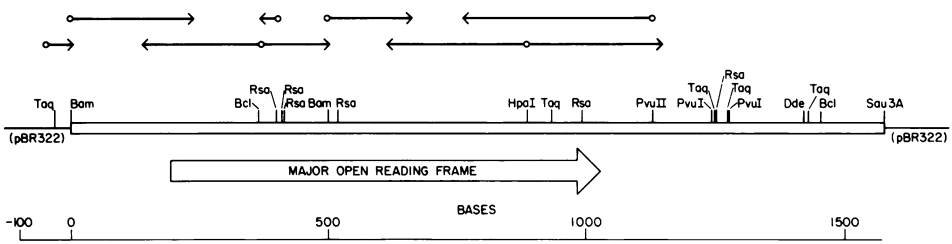

Figure 3: Strategy employed in sequencing of Eco dam region. Directionality and extent of sequence determination (see Methods section) from restriction termini are indicated by the small arrows above the restriction map. Open circles correspond to the start of 5'-end labeled DNA fragments. Sequencing from the BclI site (370) used DNA fragments isolated from pxdam1, whereas sequencing from all other sites used pdam117 DNA (Fig. 2). Location and orientation of the major open reading frame containing the dam gene is indicated by the boldfaced arrow. Both strands have been sequenced extensively and covered in many areas by duplication, although the duplications have not been depicted on this map.

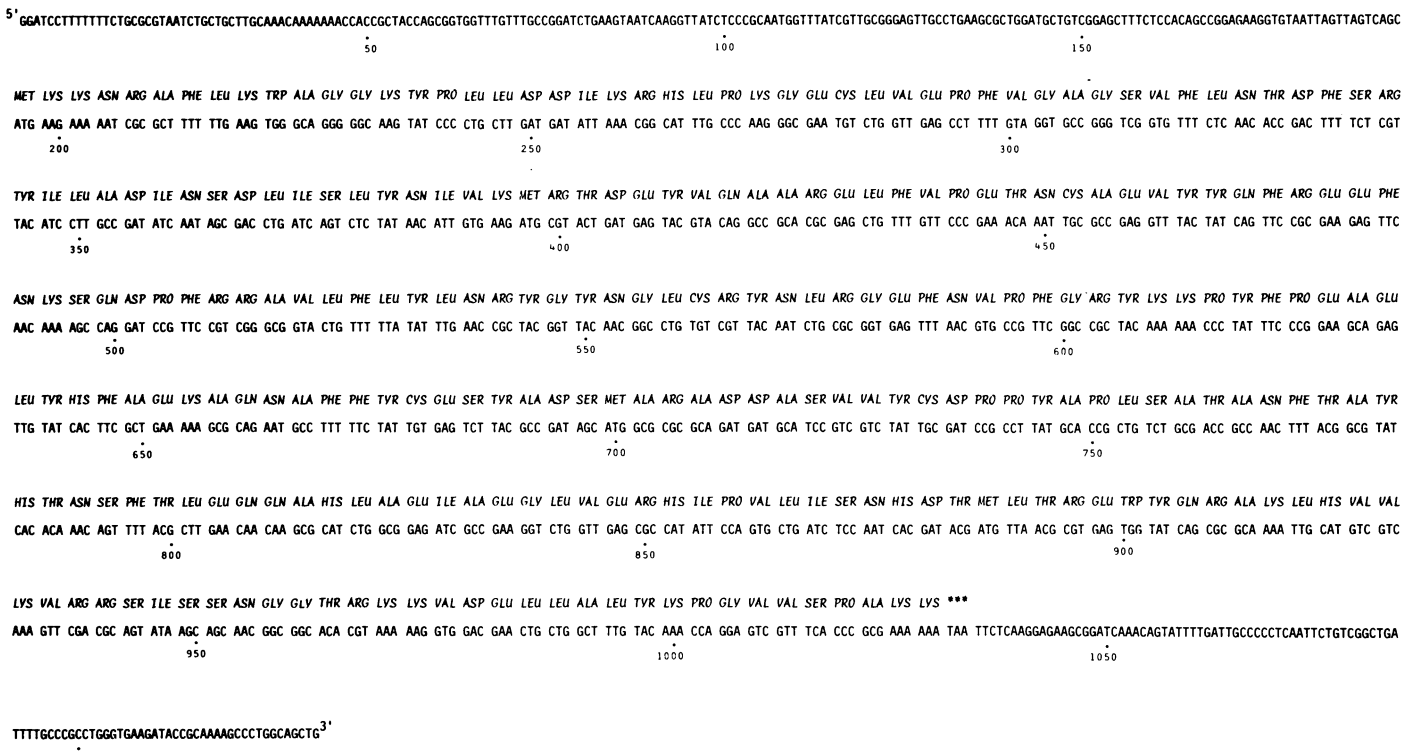

Figure 4: Nucleotide sequence of the pdam118 insert. The dam methylase gene is predicted to begin at nucleotide 195 and end at nucleotide 1029, giving rise to a protein of 31,000 MW. Also represented, above the nucleotide sequence, is the predicted amino acid sequence of the dam protein.

TABLE 1

Comparison of the Predicted Vs. the Empirically-derived Amino Acid Sequence of the dam Protein

Amino Acid	Prediction from Nucleotide Sequence (%)		Empirical Determination (%)[a]	
Alanine	9.3		9.4	
Arginine	6.8		7.2	
Asparagine	5.4	10.1	10.5	(ASX)
Aspartic Acid	4.7			
Cysteine	1.8		ND[b]	
Glutamine	2.5	9.3	9.5	(GLX)
Glutamic Acid	6.8			
Glycine	4.7		5.3	
Histidine	2.5		2.9	
Isoleucine	3.2		3.0	
Leucine	9.3		9.5	
Lysine	6.5		7.7	
Methionine	1.4		2.5	
Phenylalanine	6.1		6.0	
Proline	5.0		5.1	
Serine	5.4		4.5	
Threonine	3.6		3.4	
Tryptophan	0.7		ND[b]	
Tyrosine	7.2		6.8	
Valine	6.8		6.4	

[a] Modrich and Herman, personal communicaton.
[b] ND, not determined.

contain homologous DNA adenine methylase genes. There were no cases found in which a bacterium contains DNA sequences homologous to the pdam118 insert but has no active dam methylase. There are two cases (Moraxella bovis and Anabaena variabilis) in which the bacterium's DNA was resistant to MboI cleavage but showed no sequence homology to the pdam118 insert, even under nonstringent hybridization conditions.

DISCUSSION

(a) Identification of the dam gene.

As stated previously, the nucleotide sequence of pdam118 in conjunction

TABLE 2
Occurrence of dam Genes in Other Organisms

Organism	Hybridization to pdam 118 (dam+ clone)	Cleavage by MboI enzyme
Agrobacterium tumefaciens	–	+
Anabaena variabilis	–	–
Bacillus caldolyticus	–	+
Bacillus globigii	–	+
Enterobacter aerogenes	+	–
Enterobacter cloacae	+	–
Erwinia carotovora	+	–
Haemophilus gallinarum	+	–
Haemophilus parahaemolyticus	+	–
Haemophilus parainfluenzae	+	–
Klebsiella pneumoniae	+	–
Moraxella bovis	–	–[a]
Proteus vulgaris	+	–
Providencia stuartii	+	–
Pseudomonas aeruginosa	–	+
Rhizobium meliloti	–	+
Salmonella typhimurium	+	–
Serratia marcesens	+	–
Staphylococcus aureus 3A	–	+
Xanthomonas holcicola	–	+
Xanthomonas malvacearum	–	+
Xanthomonas oryzae	–	+

[a] Moraxella bovis differs from the other dam+ organisms listed above by having a corresponding Type II restriction endonuclease activity.

with the phenotypes of the various subclones has allowed us to set a unique set of boundaries for the dam gene. The agreement between the predicted amino acid composition of the putative protein and the amino acid composition of the purified protein also lends further support to our having correctly identified and sequenced the structural gene for the Eco dam methylase. Nevertheless, there still existed a possibility that the function cloned and sequenced was an E. coli regulatory protein that activated the dam gene rather than the methylase gene itself.

Recent experiments exclude this possibility. The HindIII-PvuII fragment of pdam118, containing the putative dam gene, was ligated to a yeast-E. coli shuttle vector, YEP228 (J. Hicks, unpublished observation) and the construct used to transform competent Saccharomyces cerevisciae cells. Chromosomal and plasmid DNA from these transformants were examined for the presence of dam modification. We have determined that all the MboI sites on the expression plasmid and as much as 30% of the chromosomal DNA is protected from MboI

TABLE 3
E. coli Codon Usage (in frequency per 1000)

		dam	RIM[a]	RIR[a]	MIX[b]
Arg	CGU	22	0	4	30
	CGC	36	3	0	21
	CGA	4	3	7	3
	CGG	7	0	0	4
	AGA	0	31	22	5
	AGG	0	3	18	2
Leu	CUU	11	16	22	18
	CUC	7	3	11	5
	CUA	0	16	14	7
	CUG	47	9	0	47
	UAA	7	28	40	21
	UUG	22	16	11	6
Ser	UCU	11	25	29	17
	UCC	7	0	4	13
	UCA	4	9	25	8
	UCG	4	12	7	9
	AGU	11	12	7	7
	AGC	18	12	14	9
Thr	ACU	4	9	18	21
	ACC	7	0	0	22
	ACA	11	16	14	5
	ACG	14	3	4	10
Pro	CCU	7	19	18	5
	CCC	18	0	0	4
	CCA	7	16	4	7
	CCG	18	0	0	19
Ala	GCU	11	12	32	37
	GCC	29	3	4	20
	GCA	25	9	14	31
	GCG	29	6	4	23
Gly	GGU	14	19	32	33
	GGC	22	9	4	29
	GGA	4	9	18	4
	GGG	7	16	22	6
Val	GUU	22	40	29	28
	GUC	18	3	7	9
	GUA	11	9	18	21
	GUG	18	6	4	17
Lys	AAA	43	68	61	46
	AAC	22	40	18	18
Asn	AAC	32	28	11	25
	AAU	22	59	61	10

Gln	CAA	7	12	25	13
	CAU	14	16	14	16
Glu	GAA	29	31	36	37
	GAG	40	31	25	18
Asq	GAC	11	6	11	27
	GAU	36	65	54	25
Tyr	UAC	32	16	7	12
	UAU	40	50	22	14
Cys	UGC	7	12	0	6
	UGU	11	9	4	5
Phe	UUC	25	22	4	18
	UUU	36	50	36	18
Ile	AUA	4	22	43	5
	AUC	18	6	14	32
	AUU	11	43	25	24
Met	AUG	14	6	25	22
Trp	UGG	7	6	7	12

[a] See references: 50 and 51.
[b] See references: 38. This is a collection of the codon usage for a total of 25 E. coli genes.

cleavage and susceptible to DpnI cleavage (R. Kostriken, et.al., unpublished observations.) (DpnI cuts DNA at the sequence GATC but only when the adenines within this site are methylated (36). Since yeast DNA is known to be devoid of N^6-methyladenine in its native state (D. Swinton and S. Hattman, personnal communication), the dam methylase activity must come from the pdam118 insert. The phenotypic effects of the adenine methylation in the yeast transformants are now being investigated.

(b) Location of dam on the E. coli chromosome.

Clones containing the dam gene were originally isolated on the basis of their proximity to the trpS gene (37). The trpS gene, coding for the tryptophanyl tRNA synthetase enzyme has also been isolated and its sequence determined (37,38). A comparison of restriction maps made of clones containing trpS and dam genes respectively, show the genes are proximal. They are separated by approximately 1 kb of DNA and are both transcribed counterclockwise on the E. coli chromosome (38).

(c) Presence of the dam gene in other bacteria.

As shown in Table 2, all members of the family Enterobacteriaceae and

also the unrelated genus Haemophilus that were tested were found to possess a functional dam methylase, both by resistance of the DNA to MboI cleavage and sequence homology to the cloned dam gene. In no instance did an organism whose DNA had sequence homology to the Eco dam gene contain DNA sensitive to MboI cleavage. There were two cases in which a bacterial DNA was resistant to MboI cleavage but the DNA showed no sequence homology to the pdam118 insert. The first case is that of Moraxella bovis, which is unique among the bacteria surveyed in that it has a Type II restriction modification system recognizing the sequence GATC and is blocked by adenine methylation within that sequence (39,40). The MboI methylase, which is part of a restriction modification system, may differ in origin as well as in function from the Eco dam methylase, and therefore lack any sequence homology. The second case is that of Anabaena variabilis, a cyanobacterium which possesses three known restriction modification systems (41,42). However, no combination of DNA methylation associated with any of these three systems could confer protection to the GATC sequences. Three possible explanations can be given for the presence of a ''dam-like'' activity but the absence of hybridization to Eco dam in Anabaena. First, it is possible that the methylase is part of a restriction modification system which is expressed at a very low level and, like the MboI methylase, is unrelated to the Eco dam. Second, it is possible that the Anabaena methylase represents a new class of adenine methylases recognizing GATC that is functionally and evolutionarily unrelated to either dam or MboI methylases. The third possibility is that the Anabaena methylase is, in fact, evolutionarily related to Eco dam but has undergone sufficient genetic drift so as to no longer hybridize, even under nonstringent conditions.

A third case worthy of mention is that of Staphylococcus aureus 3A. This bacterium also has a restriction system, Sau3A, specific for GATC (43); however, unlike the MboI system, its restriction activity is not blocked by methyladenine within the recognition sequence (16). Staphylococcus aureus 3A DNA is not resistant to MboI cleavage, nor does it contain any sequence homology to pdam118. The hybridization experiments do not indicate any sequence homology exists between the dam gene and modification methylases that belong to restriction systems.

The fact that all the bacteria tested belonging to the family Enterobacteriaceae have a dam methylase is particularly interesting in light of the reports concerning the nucleotide sequences present at their origins of replication (ori). In addition to E. coli, (12,13) origins of replication

for Salmonella typhimurium (44), Enterobacter aerogenes (45), Klebsiella pneumoniae (45), Erwinia carotovora (45), and Vibrio harveyi (Zyskind et al., submitted for publication) have been cloned and sequenced. In all six bacteria, within the approximately 250 bases necessary for oriC function, there are between 11 and 14 occurrences of the dam site, GATC. By random occurrence this sequence would be expected only once in 256 bases. Zyskind and Smith (44) proposed that the high concentration of dam sites in the ori region may be necessary for its sequence conservation. Since the post replication mismatch repair system may act by discriminating between methylated and unmethylated DNA strands, frequent occurrence of the GATC sequence could result in localization of repair enzymes within this region. Therefore, errors introduced during replication would have a greater chance of being repaired.

There is also evidence that dam methylation plays a role in the expression of the mom gene in bacteriophage Mu (46). Unlike the role as repressor of gene activity that methylation is proposed to play in eukaryotes (47,48), dam methylation seems to activate the mom function. One hypothesis currently being investigated is that dam methylation is required for transcription of the mom gene (49, R. Kahmann, pers. comm.). Having the dam gene cloned will undoubtedly assist in understanding its various roles repair, repair and transcription.

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