

Short Communications

A simple and rapid method to obtain substitution mutations in *Escherichia coli*: isolation of a *dam* deletion/insertion mutation

(Methyltransferases; genes; bacteria; recombinant DNA; methylation; conjugation)

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SUMMARY

We describe the isolation of a strain of *Escherichia coli* bearing a deletion/insertion (i.e., a substitution mutation) in the *dam* gene (*dam*-16). The mutagenesis protocol used should be applicable to any cloned non-essential gene of *E. coli*. The substitution mutation confers resistance to kanamycin and can easily be transferred to other strains by standard genetic techniques. The amount of Dam methyltransferase (MTase) in *dam*-16 strains as determined either in vitro or in vivo is below the level of detection. We conclude that the Dam MTase is not required for viability of *E. coli*.

INTRODUCTION

The *dam* gene of *Escherichia coli* specifies a DNA adenine methyltransferase (MTase), which modifies adenine residues in 5'-GATC-3' sequences in

double-stranded DNA (Marinus, 1984; 1987a,b). The Dam MTase is the only enzyme in *E. coli* known to modify this sequence. The *dam*-3 (Marinus and Morris, 1973) and *dam*-4 (Marinus and Konrad, 1976) mutations have been used extensively and

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Abbreviations: aa, amino acids; Ap, ampicillin; 2-AP, 2-amino-purine; bp, base pair(s); Cm, chloramphenicol; CGSC, *E. coli* Genetic Stock Center, Department of Biology, Yale University, New Haven CT 06510 USA; *dam*, gene coding for Dam; Dam, DNA adenine methyltransferase; Del or Δ , deletion; Hfr, high-

frequency chromosome transfer; kb, kilobase(s) or 1000 bp; Km, kanamycin; m⁶A, N⁶-methyladenine; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MTase, methyltransferase; Mud, defective Mu phage; Oc, ochre; *oriC*, origin of *E. coli* chromosome replication; PO, point of origin of Hfr transfer; ^R, resistance; ^S, sensitivity; SDS, sodium dodecyl sulfate; Sm, streptomycin; SSC, 0.15 M NaCl, 0.0015 M Na₃ citrate, pH 7.6; Tn, transposon; ::, novel joint (fusion); [], designates plasmid-carrier state.

were isolated after MNNG and ethyl methane sulfonate treatment, respectively. Strains containing these two mutant alleles do not contain detectable DNA adenine MTase activity in vitro (Marinus and Morris, 1973; Marinus and Konrad, 1976) or in vivo (Lacks and Greenberg, 1977; Bale et al., 1979). An initial observation that there was residual enzyme activity in vivo (Marinus and Morris, 1973) was subsequently shown to be due to contaminating m⁶A from RNA (Bale et al., 1979).

Two other mutant alleles were obtained by insertion of MudII (*lac* Ap^R) and Tn9 into the coding region of the *dam* gene to give *dam*-12 and *dam*-13, respectively (Marinus et al., 1983). Analysis by DNA hybridization (Southern, 1975) verified that the *dam* gene had been disrupted by the Mud insertion (Marinus et al., 1983). Cells containing these insertions did not contain detectable DNA adenine MTase activity in vitro or in vivo. Since insertions in the coding region are expected to produce an inactive protein these results led to the conclusion that the *dam* gene product was not essential for viability (Marinus et al., 1983).

In contrast to the above, Smith et al. (1985) using a different technique detected residual Dam methylation in strains containing all the *dam* mutant alleles described above. The residual methylation was preferentially located at the *oriC* region as determined by the ability of various restriction endonucleases (*Dpn*I, which cuts at methylated sites; *Mbo*I, which attacks unmethylated sites and *Sau*3A, which cleaves at both methylated and unmethylated sites) to digest *oriC* plasmid DNA extracted from Dam⁻ cells. In contrast, Szyf et al. (1982) showed that there was no detectable DNA adenine methylation in the *oriC* region of a *dam*-3 strain as determined by DNA hybridization (Southern, 1975) using an *oriC* probe after digestion of DNA with *Dpn*I or *Mbo*I.

The results described above can be explained by assuming that either another MTase exists in *E. coli* which preferentially modifies some *dam* sites at *oriC* or that there is residual Dam MTase activity in all *dam* mutants. To clarify the situation, we decided to isolate a *dam* deletion mutation which if obtained would rule out the possibility of residual Dam activity.

EXPERIMENTAL AND DISCUSSION

(a) Strategy

We have isolated a strain with a substitution mutation in the *dam* gene in two steps. First, a drug resistance gene was substituted for part of the *dam*-coding region in a plasmid, and second, the substitution mutation was recombined into the *E. coli* chromosome. In order to transfer mutations from a plasmid to the chromosome, integration of plasmids with ColE1 origins of replication into the chromosome of *polA* mutants is often used (Gutterson and Koshland, 1983). This method, however, yields a duplication of the recombined gene and also because *dam polA* strains are non-viable (Marinus and Morris, 1974), this approach was not used. In a previous study (Marinus et al., 1983), we circumvented this difficulty by transforming *recB recC sbcB* multiple mutants with linearized plasmid DNA in order to isolate the *dam*-13::Tn9 derivative. In the current study, we describe a gene replacement technique which is as simple and can be used for most cloned non-essential genes.

(b) Isolation of the chromosomal substitution mutation

To isolate a deletion in the *dam* gene on a plasmid, we used pMQ133 which contains the 854-bp *dam* gene in a 1.85-kb fragment of chromosomal DNA (Fig. 1). The *dam* gene contains unique *Eco*RV and *Hpa*I sites. The 500-bp *Eco*RV-*Hpa*I fragment was removed from pMQ133 and replaced with a 1.1-kb fragment of DNA coding for Km^R derived from Tn903 (Oka et al., 1981; a gift of M. Susskind via A. Poteete) to yield plasmid pMQ192 (Fig. 1). Since the *dam* promoter region is intact in pMQ192, a truncated polypeptide should be produced containing the 54 N-terminal aa plus two additional out-of-frame aa residues.

Plasmid pMQ192 was introduced into an Hfr strain (KL14) which transfers the *dam* gene as an early marker. Within this population, recombination should occur such that the *dam*-16 allele from the plasmid replaces the *dam* gene on the chromosome (Fig. 1). We detected such recombinants after mating with an appropriate F⁻ recipient by selecting for Km^R cells which were Dam⁻ and Cm^S. The fre-

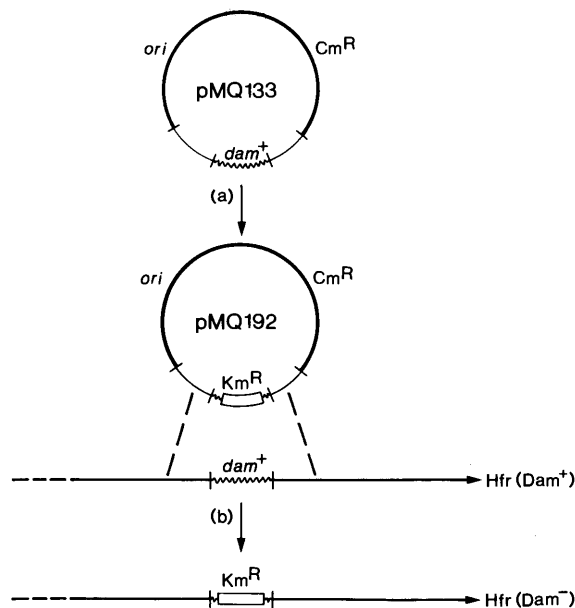


Fig. 1. Construction of a chromosomal substitution mutation in the *dam*-gene. (a) Plasmid pMQ133 was digested with *EcoRV*-*HpaI* and the 500-bp fragment so generated replaced with a 1.1-kb fragment conferring *Km* resistance. The resulting plasmid, pMQ192, was transferred to strain KL14, an Hfr cell which transfers the *dam*-gene as an early marker. (b) Recombination between plasmid and chromosome can generate a recombinant in which the chromosomal allele has been replaced by that on the plasmid. Such recombinants can be detected upon subsequent transfer of the marker to a suitable recipient cell.

quency with which such recombinants arise should be lower than that due to co-integrate formation between the whole plasmid and the chromosome (Park and Hazelbauer, 1986). After mating, the co-integrates will yield cells which are *Km*^R, *Cm*^R and *Dam*⁺. Hence, these can easily be differentiated from the desired recombinant class.

Accordingly, Hfr GM2806 (*Sm*^S) at 10^7 cells/ml was mated with the F⁻ strain AB1157 (*Sm*^R) at 10^8 cells/ml for 2 h, blended on a vortex mixer for 1 min, and plated on medium selective for *Km*^R *Sm*^R recombinants. The frequency of *Km*^R *Sm*^R colonies was 10^{-4} per input Hfr cell. Of 214 colonies analyzed, two had the desired phenotype (*Km*^R *Dam*⁻ *Cm*^S). One of these was designated GM3819 (*dam*-16). The remainder had the phenotype *Km*^R *Dam*⁺ *Cm*^R, as expected from recombinants arising by co-integrate formation.

(c) Characterization of the *dam*-16 mutation

If the *dam*-16 allele has been recombined into the chromosome, then the *Dam*⁻ and *Km*^R phenotypes should co-segregate in genetic crosses. A previous study (Marinus, 1973) has shown that *dam* is closely linked to *trpS* and *aroB*. We prepared a P1vir lysate on GM3819 (*dam*-16) and transduced *trpS*10330- and *aroB*354-containing strains to prototrophy. The results in Table I indicate that the frequency of *Km*^R linkage to *aroB* (89%) and *trpS* (91%) is that expected if the mutation is located in the *dam* gene. To monitor the *Dam* phenotype of the transductants we screened the isolates for sensitivity to 2-AP and for susceptibility of chromosomal DNA to *MboI*

TABLE I

Plasmids and strains of *E. coli* K-12 used in this study

Strain	Sex	Genotype	Source
AB2847	F ⁺	<i>aroB</i> 351 <i>mal</i> -354 <i>tsx</i> -354	CGSC
KL14	Hfr(PO68)	<i>thi</i> -1 <i>relA</i> 1	K.B. Low
GM2806	Hfr(PO68)	KL14[pMQ192]	This study
AB1157	F ⁻	<i>thr</i> -1 <i>ara</i> -14 <i>leuB</i> 6 Δ (<i>gpt</i> - <i>proA</i>)62 <i>lacY</i> 1 <i>tsx</i> -33 <i>supE</i> 44 <i>galK</i> 2 <i>hisG</i> 4(Oc) <i>rfb</i> -1 <i>mgl</i> -51 <i>rpsL</i> 31 <i>kdgK</i> 51 <i>xyl</i> -5 <i>mtl</i> -1 <i>argE</i> 3 <i>thi</i> -1	E.A. Adelberg
GM3819	F ⁻	As AB1157 but <i>dam</i> -16(Del; <i>Km</i> ^R)	This study
TrpS	F ⁻	<i>trpS</i> 10330	C.Yanofsky

Plasmid	Description
pMQ133	A 6.8-kb plasmid containing (i) the <i>dam</i> gene of <i>E. coli</i> on a 1.8-kb <i>PstI</i> - <i>SalI</i> fragment, (ii) a 1.9-kb <i>PstI</i> fragment from Tn9 conferring resistance to <i>Cm</i> , and (iii) the larger <i>SalI</i> - <i>PstI</i> fragment of pBR322 (Fig. 1a).
pMQ192	As pMQ133 but deleted for the <i>EcoRV</i> - <i>HpaI</i> fragment within the <i>dam</i> gene which has been replaced by a 1.1-kb fragment conferring resistance to <i>Km</i> (Fig. 1a).
pMQ193	A derivative of pBR322 containing a 1.1-kb <i>PstI</i> fragment conferring resistance to <i>Km</i> cloned into the <i>PstI</i> site of pBR322.
pMQ195	A derivative of pBR322 containing the <i>EcoRV</i> - <i>HpaI</i> fragment of the <i>dam</i> gene cloned into the <i>EcoRV</i> site of pBR322.

digestion. In every case Km^R recombinants were Dam^- .

Extracts of GM3819 (*dam-16*) contained no detectable *Dam* DNA MTase activity (less than 1% of the wild-type activity as determined by the method described previously; Marinus and Morris, 1973) using Dam^- unmethylated pBR322 DNA as substrate. In addition, there was no detectable m^6A in DNA in vivo as determined by the method of Bale et al. (1979). Furthermore, chromosomal DNA isolated from GM3819 was digested with *Sau3A* and *MboI* but not with *DpnI*, whereas DNA from AB1157 was digested with *Sau3A* and *DpnI* but not *MboI* (data not shown). In sum, we could detect no *Dam* methylase activity either in vitro or in vivo in GM3819 (*dam-16*).

Lack of *Dam* activity should correlate with loss of the *EcoRV-HpaI* portion of the *dam* gene in *dam-16* mutants. Fig. 2 shows that the *dam EcoRV-HpaI*

fragment from pMQ195 (Table I) does not hybridize to digested DNA from strain GM3819 (*dam-16*), but does anneal to digested DNA from AB1157 (*dam^+*).

TABLE II

Distribution of markers in transductional crosses involving *dam-16*, *aroB* and *trpS*^a

Cross	Selected markers		Unselected markers	
	<i>AroB</i> ⁺	<i>TrpS</i> ⁺	Km^R	Dam^-
<i>dam-16</i> × <i>aroB354</i>	100%	—	89%	89%
<i>dam-16</i> × <i>trpS10330</i>	—	100%	91%	91%

^a A P1_{vir} lysate of GM3819 (*dam-16*; Km^R) was prepared and used to transduce *aroB* and *trpS* mutants to prototrophy. One hundred prototrophic recombinants from each cross were then scored for Km^R and the *Dam* phenotype. *Dam* mutants are more sensitive to 2-AP than wild-type strains and their DNA is digested by *MboI*.

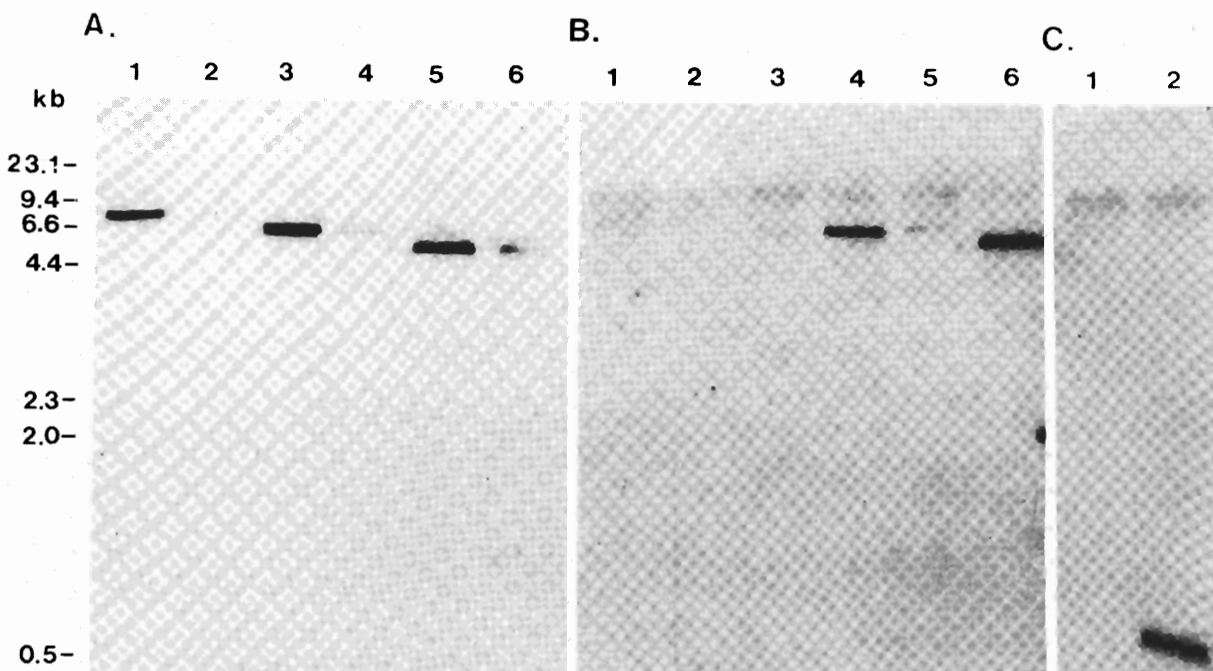


Fig. 2. Southern blot analysis of the chromosomal *dam* gene region. Panel A: DNA was isolated from AB1157 (*dam*⁺) (lanes 1, 3, 5) and GM3819 (*dam-16*) (lanes 2, 4, 6), digested with *SalI* (1, 2), *KpnI* (3, 4) or *EcoRI* (5, 6) and electrophoresed in 1% agarose. After transfer to a nitrocellulose membrane the chromosomal DNA was probed with the purified [³²P]*EcoRV-HpaI* fragment from pMQ195 for 18 h at 62°C (Southern, 1975). Following hybridization, the membrane was washed several times in 2 × SSC-SDS solution at 42°C prior to autoradiography. Numbers at the left margin indicate (in kb) the positions of end-labeled *HindIII* fragments of phage λ DNA. Panel B: the membrane used in Fig. 2A was washed in 0.4 M NaOH at 42°C for 30 min followed by an 2 × SSC-SDS wash to remove the probe used in panel A. The membrane was subsequently re-probed with a labeled *PstI* fragment from pMQ193 which contains the Km^R determinant followed by 2 × SSC-SDS washing and autoradiography. The 0.5 kb *SalI* fragment in lane 2 is not apparent in this exposure but is shown in panel C in a longer exposure from an identical experiment. The source of this fragment is not clear; there are no internal *SalI* sites in the gene for Km^R derived from Tn903 (Oka et al., 1981).

Conversely, the DNA fragment conferring Km^R , from pMQ193 (Table I), hybridizes to digested DNA from GM3819 (*dam*-16) but not to that from AB1157(*dam*⁺).

We found that the phenotypes we have monitored in *dam*-16 strains are similar to those described for other *dam* mutants (Marinus, 1985; 1987a,b). These include: sensitivity to 2-AP and MNNG; increased spontaneous mutation frequency; non-viability of *dam*-16 *lexA3* double mutants, and derepression of *sulA::lacZ* and *trpR::lacZ* fusions.

(d) Conclusions

We have described a useful and rapid method to facilitate gene replacement in *E. coli* which does not require the use of *polA* strains or other specialized methods (e.g., Raibaud et al., 1984). We also find that cells bearing the *dam*-16 allele are devoid of Dam methylase activity which confirms our previous conclusion that Dam activity is dispensable for viability (Marinus et al., 1983). If residual Dam methylation at *oriC* in *dam*-16 strains can be demonstrated, it must be due to some other MTase that is specific for Dam recognition sites at *oriC*.

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