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Biochemistry

Cloned restriction/modification system from *Pseudomonas aeruginosa*

(DNA endonuclease/DNA methylase/sequence recognition)

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ABSTRACT **DNA fragments from *Pseudomonas aeruginosa* carrying the *Pae*R7 restriction/modification genes have been cloned in the plasmid vector pBR322 and propagated in *Escherichia coli*. A subclone (pPAORM3.8) has been constructed that contains the complete restriction/modification system on a 3.8-kilobase DNA fragment. Digestion of the pPAORM3.8 plasmid with nuclease BAL-31 has yielded two types of clones. One type contains an active methylase gene but no active endonuclease gene; such clones will modify the DNA but not restrict the growth of incoming phage *in vivo*. The second type contains an active endonuclease gene but no active methylase gene, as judged both by *in vivo* tests and by the activity of the cell extracts *in vitro*. Although extracts of cells containing these plasmids display restriction endonuclease activity, these bacteria are unable to restrict the growth of incoming phage. Furthermore, chromosomal and phage DNA isolated from these host cells are not protected against cleavage by *Pae*R7 *in vitro*. The properties of *Pae*R7 endonuclease and methylase enzymes have also been examined. The *Pae*R7 restriction endonuclease recognizes and cleaves the sequence C ↓ T-C-G-A-G, as does *Xho* I. However, there exists a canonical *Xho* I site at 26.5% on the adenovirus 2 genome which is totally refractory to *Pae*R7 cleavage but is cut by *Xho* I. Under conditions of low salt, high glycerol, and high enzyme concentrations, a "*Pae*R7*" activity is found that is similar to that observed for *Eco*RI. Finally, evidence is presented that the *Pae*R7 methylase modifies the adenine residue within the recognition sequence.**

In many bacteria, restriction/modification systems produce strain-specific enzymes that allow host cells to recognize and destroy foreign DNA. This is accomplished by a restriction endonuclease that makes double-strand scissions at a limited number of specific sites on the DNA. In addition to restriction activity, such bacterial strains possess a corresponding DNA methylase that modifies specific adenine or cytosine residues within the sequence recognized by the nuclease. The methylation protects the cell's DNA against the action of its own restriction enzymes (1–4).

The genes for restriction/modification systems may be encoded on chromosomal, phage, or plasmid DNAs (1). The *Pae*R7 system belongs to the third category. It is encoded by a 42-kilobase (kb) plasmid, pMG7, carried in *Pseudomonas aeruginosa* (5). We were able to use the system's plasmid location to facilitate its cloning.

We have used molecular cloning to transfer the *Pae*R7 restriction/modification system into *Escherichia coli*. Furthermore, we have been able to divide the system and generate two types of subclones: those that express only the methylase gene, and those that express only the endonuclease gene. The viability of the latter class of clones is surprising because the chromosomal DNA from these clones is susceptible to *Pae*R7 cleavage. The existence of endonuclease$^+$ methylase$^-$ cells suggests that there are unknown control elements acting *in vivo*.

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MATERIALS AND METHODS

Bacterial Strains and Phage Stocks. The *E. coli* MM294 (recA$^+$, $r_K^-m_K^+$) (6) was obtained from D. Levy. *P. aeruginosa* strain PAO303 (pMG7) was provided by G. Jacoby. Phage ϕ80 was provided by D. Friedman.

DNA Preparations. Plasmid DNA used in the cloning and mapping experiments was isolated by the cleared lysate method (7) followed by banding in CsCl/ethidium bromide gradients. "Minipreparations" of plasmid DNA from clones were obtained by a modified version of the procedure of Birnboim and Dolly (8). Chromosomal DNA preparations were made by the method of Marmur (9). Adenovirus type 2 (Ad2) DNA was prepared by the method of Pettersson and Sambrook (10). pBR322 derivatives (pX164 and pX2281) containing *Xho* I linkers at positions 164 and 2,281, respectively, were supplied by T. J. Kwoh.

Restriction Enzymes and 5′ Mono- and Dinucleotide Analysis. The restriction endonucleases *Sal* I, *Hin*cII, *Bst*NI, *Eco*RI, *Bam*HI, *Hin*dIII, *Pst* I, *Nru* I, *Xho* I, and *Xma* I were obtained from New England Biolabs. Digestions using these enzymes were performed under conditions recommended by the manufacturer. *Kpn* I, *Rsa* I, *Bcl* I, and *Pae*R7 were prepared by P. Myers in this laboratory. *Kpn* I, *Rsa* I, and *Bcl* I digestions were performed in buffer containing 6 mM Tris, pH 7.9/6 mM $SHCH_2CH_2OH$/6 mM $MgCl_2$. *Pae*R7 digestions were done in 150 mM NaCl/6 mM Tris, pH 7.9/6 mM $MgCl_2$/6 mM $SHCH_2CH_2OH$ containing 100 μg of bovine serum albumin per ml (final volume, 50 μl) at 37°C for 2 hr. *Pae*R7* digestions were performed in 6 mM Tris, pH 7.9/6 mM $MgCl_2$/6 mM $SHCH_2CH_2OH$/20% (vol/vol) glycerol, with an excess of *Pae*R7 enzyme; incubation was for 12 hr at 37°C.

Calf alkaline phosphatase and T4 polynucleotide kinase (Boehringer Mannheim) were used to label the 5′ ends of DNA fragments generated by *Xho* I and *Pae*R7 as described by Chaconas and van de Sande (11). The 5′ mononucleotides and dinucleotides from 5′ ^{32}P-end-labeled *Xho* I and *Pae*R7 fragments were determined by using snake venom phosphodiesterase and exonuclease I (Sigma) followed by high-voltage electrophoresis (12).

Construction of Clones. pBR322 plasmids carrying DNA fragments from pMG7 were constructed by digesting pMG7 DNA (1 μg) with *Bam*HI and ligating the resulting fragments to phosphatase-treated *Bam*HI-cleaved pBR322 (0.1 μg) by using T4 DNA ligase. *E. coli* MM294 cells ($\approx 5 \times 10^9$) were transformed by the $CaCl_2$/heat-shock method of Cohen *et al.* (13). Transformants were selected on LB agar plates containing 100 μg of ampicillin per ml. A total of 27 transformants were

Abbreviations: kb, kilobase(s); Ad2, adenovirus type 2.

obtained; 9 contained inserts from pMG7.

pPAORM3.8 was prepared by digesting 2 μg of pPAORM38b with *Nru* I. The resulting fragments were recircularized with T4 ligase and used to transform MM294.

Further deletions were made in this subclone by treatment of the plasmid DNA with BAL-31. Ten micrograms of pPAORM3.8 DNA (Fig. 1) was linearized with either *Bam*HI or *Nru* I, and the DNA was treated with 2 units of BAL-31 nuclease (Bethesda Research Laboratories) under conditions recommended by the manufacturer. Aliquots (10-μl) were removed at intervals of 30 sec and 1, 2, 3, and 5 min. To each aliquot was added 20 μl of 100 mM EDTA to stop the reaction, and the samples were phenol treated and precipitated. The ends of the BAL-31-treated DNA fragments (2 μg) were repaired by using 1.4 units of the Klenow subfragment of DNA polymerase I (New England Biolabs) at 12°C for 45 min. In the case of pPAORM3.8 DNA opened at the *Bam*HI site, the repaired blunt-ended fragments were religated with T4 ligase by incubation at 4°C for 24 hr. With plasmids opened at the *Nru* I site, the repaired blunt-ended fragments were ligated to *Sal* I linkers (Collaborative Research, Waltham, MA; ratio of ends to linker, 1:20) and cleaved with *Sal* I, and the complementary ends of the *Sal* I site rejoined by T4 ligase to recircularize the plasmid.

Assays of Restriction and Modification Enzymes. (*a*) *In vitro assays.* Extracts were prepared from 5.0 ml of transformed or control cells to detect the presence of the restriction or modification enzymes. After low-speed centrifugation, cells were resuspended in 500 μl of 10 mM Tris, pH 7.6/10 mM $SHCH_2CH_2OH$/10 mM EDTA containing 0.5 mg of lysozyme and subjected to a cycle of freezing and thawing followed by 15 sec of sonication to disrupt the cell wall. $MgCl_2$ was added to a final concentration of 10 mM and the cell debris and DNA was pelleted by centrifugation. *Pae*R7 endonuclease activity was tested by digesting 2 μg of Ad2 with 4 μl of extract at 37°C for 10 min. The digestion products were analyzed by electrophoresis (14). *Pae*R7 methylase activity was tested by incubating (37°C; 30 min) 1 μg of Ad2 DNA with 10 μl of cell extract in a 50-μl reaction mixture containing 50 mM Tris (pH 7.5), 10 mM EDTA, 10 mM $SHCH_2CH_2OH$, and 0.1 mM *S*-adenosylmethionine. As a control the DNA was treated similarly but in the absence of *S*-adenosylmethionine. The Ad2 DNA was purified by phenol extraction and ethanol precipitation, challenged with *Pae*R7 or *Xho* I under conditions described above, and analyzed on a 1% agarose gel.

(*b*) *In vivo assays.* Transformants were screened for the ability to restrict and modify φ80 phage *in vivo*. To test for restriction, aliquots (0.1 ml) of transformant cultures were spot-tested with a dilution series from a high-titer phage stock, as described by Mann *et al.* (15). To test for phage modification, phage were picked from plaques on the transformed host and resuspended in 1 ml of 10 mM Tris, pH 7.4/5 mM $MgCl_2$/0.2 M NaCl/0.1% gelatin equilibrated with chloroform; the phage were serially diluted and spotted on both nonrestricting (MM294) and restricting [MM294 (pPAORM38b)] host cells.

Nomenclature. A standard method for naming plasmids containing restriction or modification genes has been adopted. Plasmids are described by: "p" for plasmid; the three letter abbreviation of the restriction/modification system (PAO); "R" for the restriction gene, "M" for the methylase gene, or "RM" for both genes; and a numeral for the size of the insert in kb. For example, a plasmid carrying both the restriction and modification genes from *P. aeruginosa* on a 3.8-kb insert is named "pPAORM3.8." Multiple plasmids containing the same complement of restriction or modification genes or both on the same-size insert fragment are further denoted by a letter after the size designation (see below).

RESULTS

Properties of *Pae*R7 Clones. The initial transfer of the *Pae*R7 restriction/modification system into *E. coli* was accomplished by ligating *Bam*HI fragments of pMG7 to pBR322 (Fig. 1). All clones generated were then tested for restriction and modification both *in vivo* and *in vitro*. Three types of plasmids were of interest: pPAORM42, containing the complete pMG7 (42-kb) inserted into pBR322; pPAORM38a, containing a 38-kb fragment of pMG7 ligated to pBR322; and pPAORM38b, containing the same 38-kb fragment but ligated to pBR322 in the opposite orientation. All three types of clones produced *Pae*R7 endonuclease and methylase as shown by *in vitro* assays (Fig. 2). In

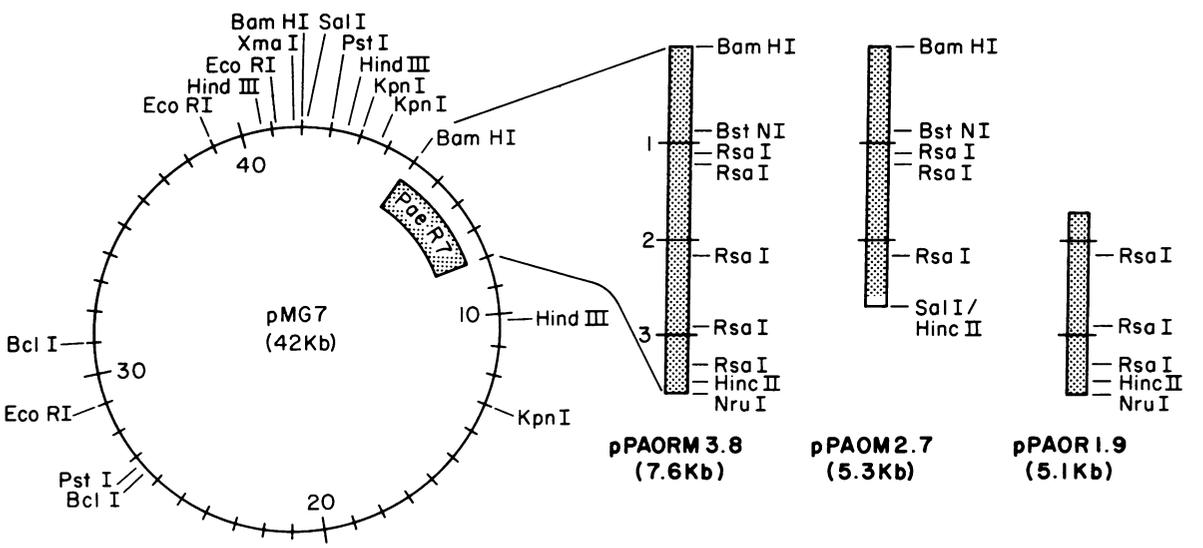

FIG. 1. Restriction enzyme maps of pMG7 and pBR322 clones containing the *Pae*R7 endonuclease or the methylase gene. The *Pae*R7 restriction/modification system is within the 42-kb pMG7 *P. aeruginosa* plasmid. Both the endonuclease and methylase genes are present in pPAORM3.8. BAL-31 digestion from the *Bam*HI site in pPAORM3.8 produced a clone (pPAOR1.9) which expressed only the endonuclease gene. BAL-31 digestion from the *Nru* I site in pPAORM3.8 yielded a clone (pPAOM2.7) which expressed only the methylase gene. The *Sal* I/*Hin*cII linker present in pPAOM2.7 is represented as a clear area. The total size of each plasmid is listed in parentheses below each construction.

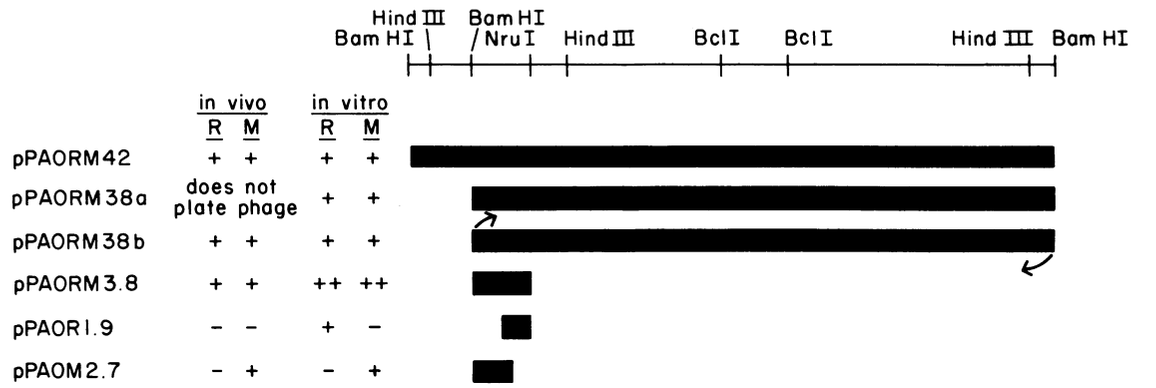

FIG. 2. Graphic summary of the pBR322 clones containing segments of pMG7. Each clone containing an insert from pMG7 was tested by *in vitro* and *in vivo* assays for the presence of the *Pae*R7 restriction/modification system. Although pPAORM38a and pPAORM38b plasmids have the same insert in opposite orientations (⇅), pPAORM38a will not plate phage but pPAORM38b will. ++, increased levels of methylase and restriction endonuclease activity.

the *in vivo* assays, pPAORM42 and pPAORM38b restricted the growth and modified the DNA of ϕ80 phage: unmodified ϕ80 had an efficiency of plating of 10^{-5} on hosts containing these plasmids compared to control MM294 host cells; modified phage had an efficiency of 1.0. Cells containing the pPAORM38a plasmid, however, were unable to produce plaques of either ϕ80 or λ phage. This was unexpected because pPAORM38a and pPAORM38b contain the same pMG7 fragment.

Subclones derived from pPAORM38a and pPAORM38b were prepared by restriction enzyme cleavage and religation to generate smaller plasmids containing the restriction/modification system. The smallest of these constructs, pPAORM3.8, contained a 3.8-kb fragment from pMG7. It both restricted and modified the DNA of ϕ80 phage as well as produced large quantities of *Pae*R7 endonuclease and methylase (Fig. 2).

To reduce further the size of the insert and to determine the order of the restriction/modification genes within this segment of DNA, the pPAORM3.8 plasmid was opened at either the *Bam*HI or *Nru* I site, treated with BAL-31 nuclease, and then religated. The smallest of the clones generated by BAL-31 treatment at the *Nru* I site contained a 2.7-kb fragment from pMG7 (Fig. 1). This plasmid, designated pPAOM2.7, made active methylase and modified the DNA of ϕ80 phage; however, it no longer restricted incoming phage, nor did it contain endonuclease as judged by an *in vitro* assay (Fig. 2). A more unusual set of clones were generated by BAL-31 treatment of *Bam*HI-cut pPAORM3.8. These clones made *Pae*R7 endonuclease but not methylase; they neither restricted nor modified the DNA of ϕ80 phage (Fig. 2). The smallest of these constructs contained a 1.9-kb insert and is designated pPAOR1.9 (Fig. 1). A composite of the two sets of BAL-31 clones indicated the order (from the *Bam*HI site to the *Nru* I site) of the genes to be first the methylase and then the endonuclease gene.

The endonuclease$^+$ modification$^-$ pPAOR1.9 clone was characterized in several ways. It was determined whether chromosomal DNA from the pPAOR1.9-containing clone was protected against cleavage by *Pae*R7 (or its isoschizomer, *Xho* I). Chromosomal DNA isolated from the pPAOR1.9 clone was sensitive to cleavage by *Pae*R7 or *Xho* I (not shown) as was DNA from ϕ80 phage grown in these cells (Fig. 3). However, chromosomal and phage DNAs isolated from clones with the R^+M^+ phenotype all were modified against *Pae*R7 and *Xho* I cleavage (Fig. 3).

Despite the fact that chromosomal DNA from the pPAOR1.9 clone was unmodified, the clone was stable. There were no observable changes in plasmid structure (with respect to its restriction map) after three rounds of plasmid isolation and retransformation. In addition, secondary and tertiary transformants still produced restriction endonuclease at the same level.

Properties of the *Pae*R7 Endonuclease. It had previously been noted that *Pae*R7 and *Xho* I have a common recognition sequence, C-T-C-G-A-G (ref. 16; P. Myers, personal communication). However, the cleavage site of *Pae*R7 had not been determined. Therefore, Ad2 DNA was cleaved with either *Pae*R7 or *Xho* I; the fragments generated were labeled at their 5′ ends with ^{32}P and digested with snake venom phosphodiesterase or exonuclease I, and the products were resolved by electrophoresis. Both enzymes gave pT as the predominant terminal mononucleotide and pTpC as the terminal dinucleotide. We conclude that *Pae*R7 cleaves at the same position as *Xho* I within the sequence 5′ C↓T-C-G-A-G 3′.

Although *Pae*R7 and *Xho* I have a common recognition and cleavage site, there are other properties that distinguish the two enzymes. *Pae*R7 and *Xho* I produce disparate digestion patterns on an Ad2 DNA substrate (Fig. 4*A*). This is due to the inability of *Pae*R7 to cleave one of the *Xho* I sites on the Ad2 genome—i.e., the site located at nucleotide 9,686 (26.54%) (17). This site

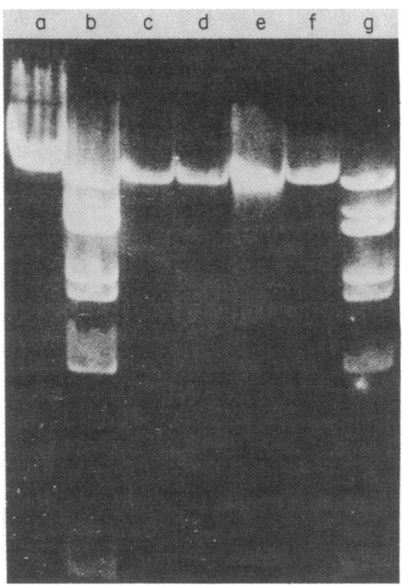

FIG. 3. A 1.0% agarose gel containing *Xho* I digests of 2 μg of ϕ80 DNAs. ϕ80 phages were grown on: *E. coli* MM294 (lanes a and b), pPAORM42 (lane c), pPAORM38b (lane d), pPAORM3.8 (lane e), pPAOM2.7 (lane f), and pPAOR1.9 (lane g). DNA in lane a was not digested.

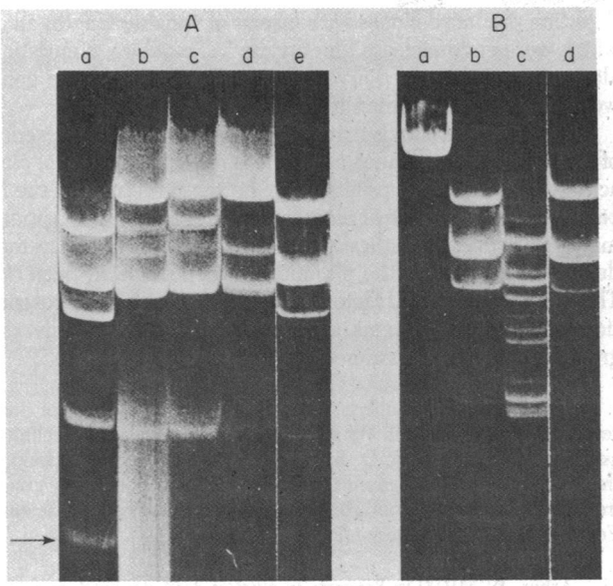

FIG. 4. Separation on a 1.0% agarose gels. (*A*) Digests of 2 μg of Ad2 DNA made with crude enzyme extracts and purified enzymes. Lanes: a, *Xho* I (purified enzyme); b, extract of PAO303 (pMG7) cells; c, extract of pPAORM3.8; d, extract of pPAOR1.9; e, *Pae*R7 (purified enzyme). Only lane a shows the presence of the smallest Ad2 fragment (→) derived from the cleavage of the site at 26.5%. (*B*) Digests of 2 μg of Ad2 DNA. Lanes a, uncut; b, *Xho* I; c, *Pae*R7*; d, *Pae*R7.

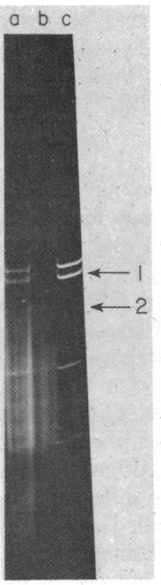

FIG. 6. A 1.5% agarose gel containing digests of pBR322 and pX2281 plasmids (having a *Xho* I linker at nucleotide 2,281). Lanes: a, pX2281 DNA modified with *Pae*R7 methylase and cleaved with *Taq* I; b, unmodified pX2281 DNA cleaved with *Taq* I; c, unmodified pBR322 DNA lacking the *Xho* I linker cleaved with *Taq* I. Fragment 1 in pX2281 DNA (lanes a and c) contained the *Xho* I linker. When modified with *Pae*R7 methylase (lane a), the plasmid was not cleaved. However, when unmodified (lane b), pX2281 DNA was cleaved (fragment 2 in lane b, and another small fragment, not shown, were generated).

was refractory to *Pae*R7 cleavage regardless of whether the DNA had been isolated from Ad2 virions or as part of an *E. coli* recombinant plasmid, indicating that blockage of cleavage is not due to DNA modification. Twenty other DNA substrates of known sequence containing *Xho* I sites all proved to be cleavable by *Pae*R7 as well as *Xho* I.

Experiments were performed with DNA containing the Ad2 26% site to determine what properties were responsible for blocking cleavage. When ligated to DNA from a different source, "hybrid" sites containing sequences from the right side (toward higher Ad2 map positions) of the 26% site were cleavable by both *Xho* I and *Pae*R7. However, a hybrid site containing sequences to the left (toward lower map positions) of the original 26% site still was resistant to *Pae*R7 (Fig. 5).

During efforts to optimize digest conditions for *Pae*R7, a second endonucleolytic activity was observed in *Pae*R7 preparations. This activity was found under conditions of low salt, high glycerol, and high enzyme concentrations with prolonged incubation (Fig. 4*B*). The second activity was inhibited by high salt (150 mM NaCl). Under identical digestion conditions, *Xho* I showed no secondary activity.

Specificity of the *Pae*R7 Methylase. We determined which base is methylated within the *Pae*R7 recognition site by the method described by McClelland (18). Because the central tetranucleotide of the *Pae*R7 recognition site, T-C-G-A, is also the *Taq* I recognition site (19), all *Pae*R7 sites are also *Taq* I sites. It had been shown previously that *Taq* I cleavage is blocked by N^6-methyladenine within its recognition site but is insensitive to the presence of 5-methylcytosine (18, 20). Therefore, failure of *Taq* I to cleave a *Pae*R7 site after it had been modified by the *Pae*R7 methylase would indicate that this enzyme is a DNA adenine methylase. *Taq* I could not cleave a *Pae*R7 site (inserted as a *Xho* I linker into pBR322) after having been modified by the *Pae*R7 methylase (Fig. 6). This result strongly suggests that the *Pae*R7 methylase acts at the recognition site to produce 5′ C-T-C-G-^{m}A-G 3′.

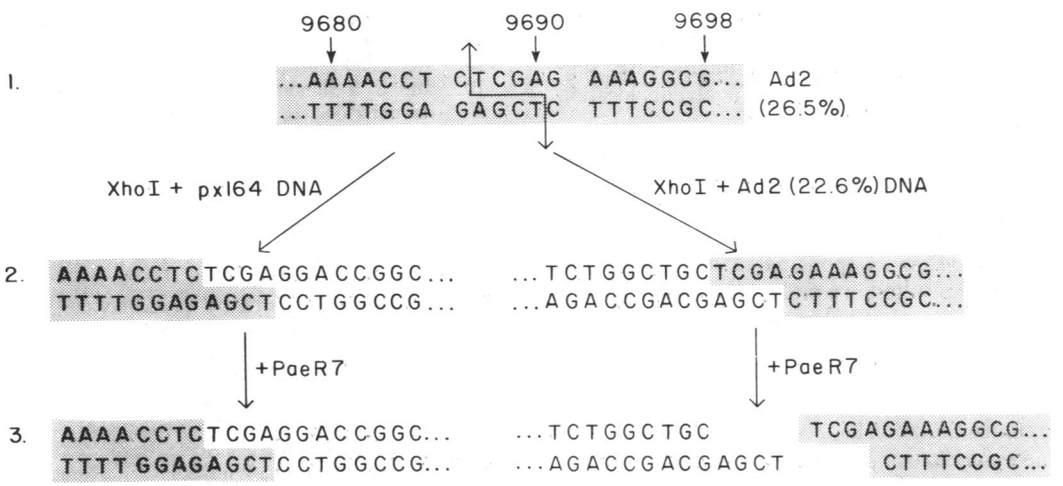

FIG. 5. Constructions of hybrid *Pae*R7 sites by using 26.5% site on the Ad2 genome (nucleotides 9,679–9,698). The 26.5% site was cleaved with *Xho* I and the two ends (stippled) were religated to other *Xho* I sites (pX164 is pBR322 plasmid with a *Xho* I linker at position 164). After the hybrid sites were constructed, they were digested with *Pae*R7. Sequences 5′ to the 26.5% site (toward lower nucleotide numbers) in Ad2 DNA are responsible for the resistance of this site to cleavage.

We have also noted that *Pae*R7 methylation can block cleavage of its recognition site by *Xho* I and *Ava* I (C-Py-C-G-Pu-G). However, this does not necessarily imply that these two enzymes have the same cognate methylation site (21).

DISCUSSION

Efforts to clone the genes for various restriction/modification systems in *E. coli* have met with success in only four cases: *Eco*RI (22), *Eco*RII (23), *Hha* II (15), and *Pst* I (24). It is uncertain why attempts to clone other systems have not succeeded. The fact that *Pae*R7 is a type II system (25) which is plasmid-borne (5) facilitated the isolation and transfer of the genes. Clones containing the *Pae*R7 system also have been reported by Theriault and Roy (26).

*Pae*R7 possesses some interesting properties. The inability of this enzyme to cleave the Ad2 26.5% site is a phenomenon which, to our knowledge, is unique (27, 28). Although many restriction endonucleases are known to cleave different substrate sites at variable rates dependent on their flanking sequences (3, 27, 28), they eventually do cleave all canonical sites. In the case of *Pae*R7, there clearly is an effect induced by the sequence 5′ (toward lower nucleotide numbers) to the Ad2 *Pae*R7 site which renders the site completely refractory. Computer searches of the sequences up to 150 nucleotides 5′ to this *Pae*R7 site have not shown the presence of any regions that could form secondary structures of significant stability. This, of course, does not preclude the possibility of interactions between distal regions leading to the formation of a stable structure. Alterations in the primary flanking sequences will be required to determine the cause of this inhibition.

The most unusual feature of the *Pae*R7 clones is the finding that a functioning restriction enzyme gene can be present in the cell without an accompanying methylase gene. In other restriction/modification systems, such as the *Eco*RI and *Hha* II systems, the presence of the restriction enzyme gene without its corresponding methylase is lethal (ref. 29; B. Schoner and H. Smith, personal communication). Consequently, obtaining clones that are endonuclease$^+$ methylase$^-$ was unexpected. Although the mechanism that makes the *Pae*R7 clones viable is not yet known, experiments involving the pPAOR1.9 clone demonstrate that the restriction enzyme in this clone is not active *in vivo* because ϕ80 phage can grow unrestricted. There are at least three possible explanations of this phenomenon. First, it may be that the restriction enzyme is physically separated from the cell's DNA. Preliminary experiments attempting to release periplasmic proteins by subjecting the cells to osmotic shock failed to release the *Pae*R7 endonuclease, suggesting that compartmentalization does not involve its location in the periplasmic space.

A second possibility is that the *Pae*R7 restriction gene has undergone mutation and now codes for an altered protein that is nonfunctional under physiological conditions. There is a precedent for this—the occurrence of such a mutation in clones containing the *Eco*RI system. A spontaneous mutation in the endonuclease gene (30) resulted in a mutant that was unable to restrict incoming λ phage; nevertheless, extracts of the mutant cells contained high levels of endonuclease activity. The mutant form of the enzyme differs from the wild type in optimal reaction parameters (P. Greene, personal communication). To determine whether a similar mutation has occurred in the *Pae*R7 system, it will be necessary to compare the DNA sequences of the restriction gene from pPAOR1.9 (endonuclease$^+$ methylase$^-$) with the gene from pPAORM3.8 (endonuclease$^+$ methylase$^+$) clones. In this respect, it is interesting to note that BAL-31 digestion produced clones with inserts of variable length, all of which were endonuclease$^+$ methylase$^-$. Therefore, a mutation altering the *in vivo* activity of the *Pae*R7 endonuclease gene would have to be common to all of these constructs.

The third possibility is that in the *Pae*R7 system the presence of the restriction endonuclease alone is not sufficient for *in vivo* activity. Another gene product may be required to elicit the *in vivo* activity. The control element, whatever its composition, may have evolved specifically to aid this plasmid-borne system during the transfer of the plasmid between different bacteria. The presence of a third factor would imply that type II restriction/modification systems are a more complex and diverse group than has been assumed.

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