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Cloning and characterization of genes for the *PvuI* restriction and modification system

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ABSTRACT

The genes encoding the endonuclease and the methylase of the *PvuI* restriction and modification system were cloned in *E.coli* and characterized. The genes were adjacent in tandem orientation spanning a distance of 2200 bases. The *PvuI* endonuclease was a single polypeptide with a calculated molecular weight of 27,950 daltons. The endonuclease was easily detectable when the gene was expressed from its endogenous promotor and present on a low copy plasmid, but expression was considerably enhanced when the endonuclease gene was placed under the control of a strong promotor on a high copy plasmid. The methylase did not completely protect plasmid DNA from R·*PvuI* digestion until the methylase gene was placed under *lac* promotor control in a multicopy plasmid. In the absence of the M·*PvuI* methylase, expression of the R·*PvuI* endonuclease from the lac promotor on a multicopy plasmid was not lethal to wild type *E.coli*, but was lethal in a temperature-sensitive ligase mutant at the non-permissive temperature. Moreover, induction of the R·*PvuI* endonuclease under λp_L promotor control resulted in complete digestion of the *E.coli* chromosome by R·*PvuI*.

INTRODUCTION

The wide variety of restriction and modification systems among the bacteria offers insights into protein–DNA interactions and the expression of similar genes and operons in many different kinds of bacteria (for a recent review of restriction modification systems, see 1). The genes for over 100 restriction and modification systems have been partially or completely characterized (2). In the type II systems which have been characterized, the modification function is carried out by a 'methylase' which methylates adenine at the N6 position, or cytosine at the C5 or N4 position. The cleavage is carried out by an 'endonuclease', which is usually a dimer of a single polypeptide. In nearly all cases, the endonuclease and methylase genes are adjacent (2). Restriction systems are usually cloned by generating a genomic bank in a plasmid vector, and selecting plasmids with inserts that code for the methylase, and therefore are protected from endonuclease digestion (3, 4, 5). If the insert is large enough, the clones usually contain plasmid DNA which code for the methylase and endonuclease.

R·*PvuI* is a type II restriction endonuclease from *Proteus vulgaris* that recognizes and cleaves between the T and the C in the sequence 5'CGATCG3' in double stranded DNA (6). The exact specificity of the M·*PvuI* methylase is unknown, but since R·*PvuI* cleaves *dam* methylated DNA, it is not an adenine methylase (24). We cloned and expressed these genes, and report here the location and orientation of the restriction and modification genes. The M·*PvuI* methylase gene was placed downstream from a *lac* promotor and the R·*PvuI* endonuclease gene was placed downstream from a λp_L promotor. Expression of the R·*PvuI* endonuclease gene was not lethal to cells that lacked the M·*PvuI* methylase, suggesting that the R·*PvuI* endonuclease was compartmentalized away from the host chromosome, which was unmodified and susceptible to cleavage, or that the R·*PvuI*-induced damage was repaired by ligase. Evidence is presented that suggests *E.coli* is able to repair some breaks induced by R·*PvuI*, but that this repair can be overcome by large amounts of R·*PvuI* endonuclease While expression of a restriction endonuclease without protection by the corresponding methylase is often lethal (9, 10), it has been reported that *TaqI* and *Pae*R7 can be expressed without methylase protection of the host (16,17). While this manuscript was being prepared, patent applications were made public indicating the genes encoding the *PvuI* system from *Proteus vulgaris* had been cloned and expressed in *Escherichia coli* by others (7, 8).

MATERIALS AND METHODS

Bacterial strains, growth, and transformation conditions

Proteus vulgaris ATCC 13315 is a strain on deposit at the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852. *E.coli* strains were grown in LB or LB + 0.2% maltose (11). When appropriate, antibiotic supplements were 100 mg/l ampicillin (Ap), 20 mg/l tetracycline (Tc), or 25 mg/l chloramphenicol (Cm). *E.coli* strain DH10B (12) was used as host. To generate BRL3050, the lig7ts allele from *E.coli* strain N2604 (13) was transduced to a rec$^+$ DH10B derivative by Dr. Fred Bloom of Life Technologies, Inc (LTI). Competent *E.coli* strains were obtained from LTI or made by standard methods. Electroporation was as described (14). DH10B/pRK248CI host cells were used with plasmid constructions which placed the R·*PvuI* endonuclease gene under λp_L promotor control.

Endonuclease assay

Cell pellets from 35 ml cultures were resuspended in 1 ml SB (10 mM Tris pH 7.5, 10 mM β-mercaptoethanol, 1 mM EDTA) and sonicated for 30 sec. Debris was removed by centrifugation. One μl of the cell extract was added to 15 μl of substrate (50

ng λ DNA per μl in LTI REact 6). This reaction was diluted threefold serially through at least three more tubes, each of which contained 10 μl of substrate. After incubation at 37°C for 60 min, the reaction was stopped by addition of SDS and each sample was electrophoresed.

Osmotic shock

DH10B/pS58 was grown at 37°C to about 10^9 cells per ml, and 30 ml were harvested by centrifugation. The cells were resuspended in 5 ml SM + 20% sucrose and incubated at 22°C for 20 min. After centrifugation, the cells were osmotically shocked by resuspension in 0.3 ml 0.5 mM $MgCl_2$ at 4°C (16). The cells were centrifuged again, and the supernatant was assayed for activity. The pellet was resuspended in 1 ml SB, sonicated as usual, and assayed for activity.

Plasmid and phage vectors

The cosmid pCP13 (17) contains three *Pvu*I sites. Two *Pvu*I sites are located within 400 base pairs of one another, in or near the tetracycline resistance determinant. The third *Pvu*I site is in the kanamycin resistance determinant, and is removed by digestion of pCP13 with *Cla*I. Phage M13mp19 and plasmids pSPORT, pUC18 and pUC19 are commonly used cloning vectors (16, 18). The plasmid $pUCp_L$ is a derivative of the cloning vector pUC19 which contains the λp_L promotor (19). The plasmid pRK248CI is a derivative of RK2 which contains the CI857 gene which codes for a temperature-sensitive repressor that controls expression from the λp_L promotor (20). λp_L expression was induced by warming the culture to 42°C for 45 min, followed by incubation at 37°C for 3 hours.

Cloning and sequencing procedures

Enzymes were from LTI unless otherwise specified. Plasmid DNA was isolated by the alkaline lysis method (11). Genomic DNA was an unfractionated mix of plasmid and chromosomal DNA (11). A cosmid library of genomic DNA from a *P. vulgaris* strain was constructed in pCP13. Genomic DNA was partially digested with *Hpa*II endonuclease, mixed with *Cla*I-treated dephosphorylated pCP13 DNA, and the mixtures treated with ligase. Five μl of the mixture was packaged into phage lambda particles (lambda packaging kit, LTI). The lambda particles were used to infect DH10B cells which had been grown in YET-maltose, and the infected cells were plated on YET agar plates (1.5% agar) which contained 20 μg/ml tetracycline. After incubation for 18 hours at 37°C, about 50,000 colonies were obtained. Plasmid DNA was isolated from the pooled library. Half a microgram of the plasmid bank DNA was digested for 18 hours with 25 units of R·*Pvu*I. Additional R·*Pvu*I (25 units) and 1 unit of calf intestinal phosphatase was then added, and the mixture incubated for one more hour. After phenol extraction and ethanol precipitation of the digest, 50 ng of the digested bank DNA was used to transform 20 μl of DH10B recipient *E.coli* cells by electroporation. There were about 50 transformants from each transformation.

Polymerase Chain Reactions (PCR) were carried out under standard conditions (2.5 units Cetus Taq DNA polymerase, 10 ng target DNA, 25 mM Tris-HCl pH 8.3, 5 mM $MgCl_2$, 50 mM NaCl, 1 μM of each oligonucleotide, 0.2 mM each of deoxyribonucleotide triphosphates (dGTP, dATP, dTTP, and dCTP) in a thermal cycler. The regime was 1: 5 min at 94°C; 2: 15 cycles of 94°C (10 sec), 55°C (15 sec), 72°C (10 sec); 3: 10 min at 72°C). DNA fragments generated by restriction endonuclease digestion or PCR were separated by gel electrophoresis and isolated from gel slices. In some cases, vector DNA was treated with calf intestinal alkaline phosphatase (Boehringer Mannheim), and insert DNA was treated ('blunted') with T4 DNA polymerase or the large fragment of *E.coli* DNA polymerase I in the presence of deoxyribonucleotides. DNA sequence analysis was performed by dideoxy chain terminating sequencing from single-stranded and double-stranded DNA templates using modified T7 DNA polymerase from U.S. Biochemicals.

RESULTS

Cloning the *Pvu*I region

As described in the methods section, large fragments of genomic DNA from *P. vulgaris* were cloned in the cosmid pCP13. Plasmid DNA from the genomic bank was cleaved with R·*Pvu*I, and the digest was used to transform *E.coli* recipients. Plasmid DNA from transformants was digested with R·*Pvu*I or *Eco*RI and then analyzed by gel electrophoresis. The results for 6 such clones (pS51-pS57) are shown in Fig. 1. In agarose gels such as that shown in Fig.1, linear above 12 kb DNA migrates to a position which is only slightly above the 12 kb linear standard, and circular plasmids of 40−50 kb migrate about half as fast. The species which migrates only 25% as fast as large liner DNA is presumed to be an open form of the plasmid. As expected, the migration of the uncleaved DNA (pS51−pS57) was typical of preparations of pCP13 which contained large inserts and are from 40 to 50 kb in size (lanes 7, 9, 11, 13, 15, and 17). In addition to the circular DNA, the preparations contain some linear DNA which is chromosomal DNA, linearized plasmid DNA, or both. All the plasmids (pS51−pS57) were linearized by R·*Pvu*I to some extent, varying between about 50% linearization to complete linearization (Figure 1, lanes 8, 10, 12 14, 16, 18). No plasmids appear to have been cleaved by R·*Pvu*I more than once. All but one plasmid shared an 8 kb *Eco*RI fragment Fig. 1, lanes 1−6). It was apparent that most or all of the clones had the M·*Pvu*I methylase gene, but the M·*Pvu*I methylase gene was only partially active in these clones. One plasmid (pS51) was chosen for further study.

Localizing the R·*Pvu*I endonuclease gene

The subcloning of the *Pvu*I region is shown in Fig. 2. Cell extracts of DH10B/pS51 contained about 4,000 units of R·*Pvu*I per g of cells. It was reasoned that the 8 kb *Eco*RI fragment, common to most of the clones, might contain the endonuclease and methylase genes. Plasmid DNA from DH10B/pS51 was digested with *Eco*RI, and the 8 kb fragment was excised and subcloned into the plasmid pSPORT 1, forming plasmid pS57. The plasmid pS57 contained an 8 kb *Eco*R I fragment and a 700 bp *Eco*R I fragment from pCP13 in addition to the 4.1 kb pSPORT fragment. Cell extracts from a DH10B/pS57 culture contained about 2,000 units of R·*Pvu*I per gram of cells. A restriction map of plasmid pS57 was generated, using restriction enzymes *Kpn*I, *Sph*I, *Hind*III, and *Eco*RI (Fig. 2). The small *Hind*III fragment of pS57 was subcloned into the *Hind*III site of pUC19, forming plasmid pS59. Extracts from DH10B/pS59 contained about 4,000 units R·*Pvu*I endonuclease per g cells. The small *Kpn*I-*Hind*III fragment of pS57 was subcloned into the *Kpn*I-*Hind*III sites of pUC19, forming plasmid pS58. Since cell extracts from DH10B/pS58 contained about 15,000 units of R·*Pvu*I per g cells, it was clear that the R·*Pvu*I endonuclease

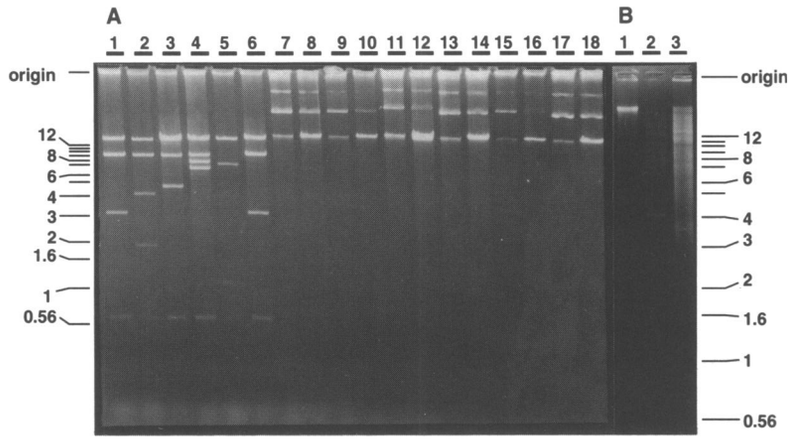

Figure 1. Agarose gels, stained with ethidium bromide after electrophoresis at 10 V/cm. The positions and sizes, in kb, of linear DNA standards are indicated for each gel. **A.** Plasmid DNA from clones pS51 through pS56, which were R·*Pvu*I-resistant cosmid clones of *P. vulgaris* genomic DNA. DNA was cleaved with *Eco*R I, R·*Pvu*I, or incubated in reaction buffer without R·*Pvu*I. 1: pS51 + *Eco*R I, 2: pS52 + *Eco*R I, 3: pS53 + *Eco*R I, 4: pS54 + *Eco*R I, 5: pS55 + *Eco*R I, 6: pS56 + *Eco*R I, 7: pS51, 8: pS51 + R·*Pvu*I, 9: pS52, 10: pS52 + R·*Pvu*I, 11: pS53, 12: pS53 + R·*Pvu*I, 13: pS54, 14: pS54 + R·*Pvu*I, 15: pS55, 16: pS55 + R·*Pvu*I, 17: pS56, 18: pS56 + R·*Pvu*I. **B.** Genomic DNA from DH10B/pS63. 1: cells grown at 30°C (uninduced for *Pvu*I), 2: same as lane 1 but isolated DNA was cleaved in vitro with R·*Pvu*I. 3: cells pulsed at 42°C and incubated at 37°C (induced for *Pvu*I).

gene lay entirely within the *Kpn*I−*Hind*III fragment of pS58. Since pS58 did not encode the M·*Pvu*I methylase (see below), R·*Pvu*I endonuclease can be produced from cells that are not protected by methylation.

Localizing the M·*Pvu*I methylase gene

Each of the plasmids pS58 through pS60 contain two *Pvu*I sites, spaced 560 bp apart, in the pUC19 portion of the plasmid. A subclone with the M·*Pvu*I methylase gene is expected to be protected from R·*Pvu*I endonuclease digestion at these sites. An expression clone (described below) which produced the M·*Pvu*I methylase from the lac promotor did completely protect these sites from R·*Pvu*I endonuclease digestion (Fig. 3, lanes 1 and 2). The *Pvu*I sites in pS59 were partially protected from digestion by R·*Pvu*I endonuclease (Fig. 3, lanes 3 and 4). Although the plasmid DNA is substantially protected from digestion with R·*Pvu*I endonuclease, the 560 bp *Pvu*I fragment and two linear forms of pS59 are evident after R·*Pvu*I digestion of pS59. In contrast, plasmid pS58 was not methylated at the *Pvu*I sites (Fig. 3, lanes 5 and 6), and therefore does not encode a functional M·*Pvu*I methylase gene. The *Pvu*I digest of pS58 DNA in Fig. 3 contains some linear plasmid, indicating that pS58 DNA was not completely cleaved by *Pvu*I. We do not believe that this indicates that methylase activity was conferred by pS58 since other digests of pS58 and similar plasmids were more complete (not shown). In most restriction-modification systems that have been described, the methylase and endonuclease genes are tightly linked (2). In light of this, and since the M·*Pvu*I methylase gene was present on the *Hind*III fragment in pS59 but not on the *Kpn*I−*Hind*III fragment in pS58, we hypothesized that the M·*Pvu*I methylase gene was closely linked to the R·*Pvu*I endonuclease gene and was probably interrupted by the *Kpn*I site.

Sequencing of the *Pvu*I methylase and endonuclease genes

Enough of the *Pvu*I region was subcloned and sequenced so that the location and orientation of the genes could be established,

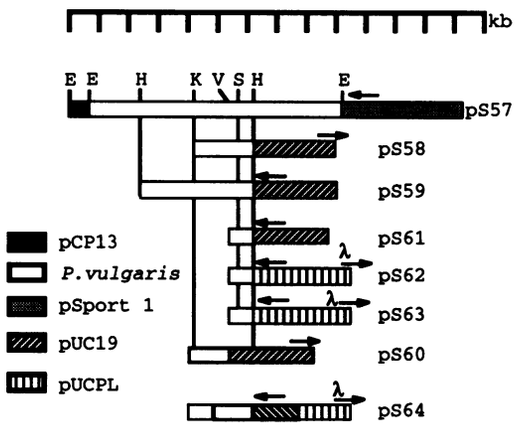

Figure 2. Restriction maps of *Pvu*I subclones. Scale in kb and source of fragments are indicated. Arrows show the direction of the lacUV5 (plain arrows) and the λp_L (marked with λ) promoters. Restriction sites marked are *Eco*RI (E), *Hind*III (H), *Eco*RV (V), and *Sph*I (S).

but the sequence was not completed, nor was it verified by sequencing in both directions. For this reason, only a short sequence (which was sequenced in both directions) is presented. The sequence that was obtained revealed three tandem open reading frames, which were easy to identify because of the high A + T content of the DNA (Fig. 4). The first two open reading frames, separated by an unsequenced region, were presumed to be part of a single gene coding for the M·*Pvu*I methylase, since this gene would be interrupted by the *Kpn*I site, and would cover the 1,200 bases typical of a methylase gene (2). Another open reading frame was presumed to be the gene coding for the R·*Pvu*I endonuclease, since it was found in the *Kpn*I-*Hind*III fragment which was sufficient to encode the R·*Pvu*I endonuclease. The R·*Pvu*I endonuclease gene was followed by an inverted repeat which may act as a transcriptional terminator (Figure 5).

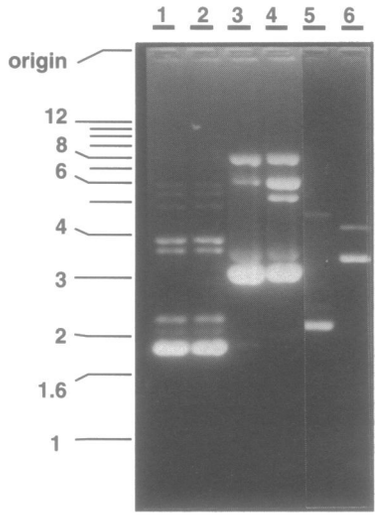

Figure 3. Agarose gel, stained with ethidium bromide after electrophoresis at 10 V/cm. Plasmid DNA cleaved with R·*Pvu*I or incubated in reaction buffer without R·*Pvu*I. 1: pS60, 2: pS60 + R·*Pvu*I, 3: pS59, 4: pS59 + R·*Pvu*I, 5: pS58, 6: pS58 + *Pvu*I.

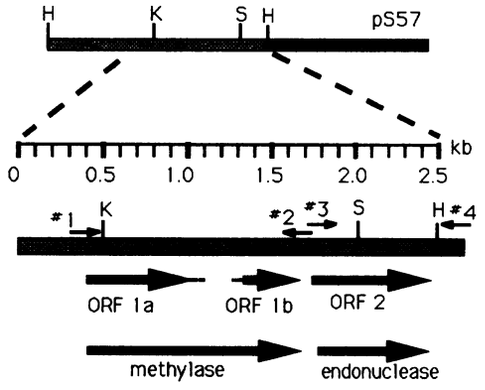

Figure 4. Location of the *Pvu*I methylase and endonuclease genes by partial sequencing and PCR. Scale in kb is indicated for the expanded region of pS57. The endonuclease was encoded by the DNA between the *Kpn*I (K) and *Hin*DIII (H) sites, and the methylase was encoded between the *Hind*III sites on pS57 (see text). Three open reading frames (ORF's) from the partial sequence are indicated. ORF 1a and 1b were separated by an unsequenced region, but were supposed to be the amino and carboxy termini of the methylase gene. The possibility that this region contains a small ORF in addition to the methylase gene was not ruled out. ORF2 was supposed to be the endonuclease gene, and this was confirmed by subcloning (see text). The locations of the oligonucleotides used to clone each gene by PCR are indicated as small arrows.

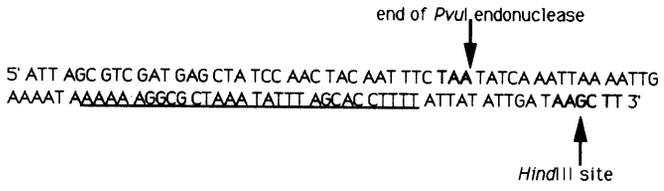

Figure 5. DNA sequence following the R·*Pvu*I endonuclease gene. The end of the R·*Pvu*I endonuclease gene and the *Hind*III site that forms the junction with the pUC19 vector are in bold type. Inverted repeats are underlined.

Expression of the *Pvu*I endonuclease and methylase

An oligonucleotide (5′ CCTAGTAACGATAGAAA 3′) upstream from the presumed methylase coding region and an oligonucleotide downstream from this region (5′TATCATTACCATCAACG 3′) were used to generate an 1100 bp DNA fragment from pS59 using the polymerase chain reaction (PCR). This fragment was treated with T4 DNA polymerase to make the ends blunt, and cloned into the *Sma*I site of pUC19, forming plasmid pS60 (Fig. 2). Plasmid DNA from DH10B/pS60 was completely resistant to R·*Pvu*I endonuclease, confirming the expression of the methylase from the region identified by the subcloning data and the open reading frame deduced from the partial sequence (Fig. 2 and Fig. 4). It was not determined that the two open reading frames ORF 1a and 1b were the the amino and carboxy termini of the methylase.

An *Nde*I site was placed at the presumed ATG start codon of the R·*Pvu*I endonuclease coding sequence. An oligonucleotide which mutagenized the appropriate sequence into an *Nde*I site (5′ TAAATACG**CATATG**GTAATGATATTGTTG 3′) was used in conjunction with an oligonucleotide corresponding to pUC19 DNA (5′ AACAGCTATGACCATG 3′) to generate a PCR fragment from pS59 containing the R·*Pvu*I endonuclease gene. This fragment was cloned into the *Nde*I and *Hind* III sites of pUC19, forming plasmid pS61 (Fig. 2). The R·*Pvu*I coding sequence represented in the small *Nde*I-*Hind*III fragment of pS61 was placed under λp_L promoter control by cloning it into the *Nde*I-*Hind*III sites of pUCp_L, forming pS62 (Fig. 2). Since DH10B/pRK248cI/pS62 did not produce R·*Pvu*I endonuclease activity, it was presumed that the R·*Pvu*I endonuclease allele isolated by PCR was mutant, and therefore the majority of the allele was replaced by cloning the small *Eco*RV−*Hind*III fragment of pS58 into the large *Eco*RV−*Hind*III fragment of pS62, forming plasmid pS63 (Fig. 2). DH10B/pRK248cI/pS63 cells had >100,000 units/g cells after heat induction of the λp_L promotor. The M·*Pvu*I methylase gene was introduced into pS63 by cloning the larger *Sca*I-*Eco*RI fragment of pS60 (in which the *Eco*RI end had been made blunt) into the smaller *Hind*III-*Sca*I fragment of pS63 (in which the *Hind*III end had been made blunt), forming plasmid pS64 (Fig. 2). It was reasoned that the inverted repeat sequence downstream from the R·*Pvu*I gene and present in pS64 may act as a transcriptional terminator and allow efficient transcription from both the lac and λp_L promoters even though they were in opposite orientation (Fig. 2 and Fig. 5). DH10B/pRK248cI/pS64 cells made more than 150,000 units of R·*Pvu*I endonuclease/g cells.

When overproduced, R·*Pvu*I cleaves the host chromosome

As described above, the clone which contained plasmid pS58 was stable without M·*Pvu*I methylase expression. It appeared that the R·*Pvu*I endonuclease did not have access to the unmodified host chromosome, which has many *Pvu*I sites. One possibility is that the R·*Pvu*I endonuclease is excreted or periplasmic. However, the enzyme was not detectable in culture supernatants, nor was more than 5% of the endonuclease activity released from cells by osmotic shock. Alternatively, it may be that the host chromosome is protected by binding proteins, or that the conditions inside the cell are not sufficient for R·*Pvu*I endonuclease activity. To test this hypothesis, we overproduced the enzyme in a host that had no M·*Pvu*I methylase. As described above, DH10B/pRK248cI/pS63 cells have no M·*Pvu*I methylase, but the R·*Pvu*I endonuclease is overproduced when the cells are induced by heating. When these cells are induced, they stop

growing as measured by optical density, and die, as indicated by a hundredfold drop in the ability to form colonies on an YET agar. Genomic DNA isolated from the induced cells was similar to a complete in vitro R·*Pvu*I digest of DNA from uninduced cells (Fig. 1B). The difference between the *in vitro* and *in vivo* digests was that the smaller fragments of the latter digest appeared to be degraded, consistent with exonuclease digestion of the R·*Pvu*I fragments after they were produced *in vivo*. The same heating regimen used to induce overexpression did not halt the growth of DH10B/pS58 cells, nor did it result in a detectable amount of R·*Pvu*I digestion of the chromosome (not shown). We conclude that the intracellular conditions are perfectly adequate for the digestion of the chromosome by R·*Pvu*I and that the mechanism which prevents R·*Pvu*I induced degradation of the chromosome is overcome when the enzyme is overproduced.

We then sought to determine if moderate amounts of R·*Pvu*I cleave the host chromosome, but at a rate that is repaired by *E.coli* DNA ligase. *E.coli* mutant BRL3050 is a DH10B derivative which is deficient in DNA ligase at 30°C and lacks detectable ligase activity at 42°C (13). BRL3050 was transformed with pRK248cI, followed by pS63, or just by pS58. Transformants, selected at 30°C, were streaked on selective agar plates and incubated for 24 hours at 30°C or 37°C. BRL3050/pS58 transformants grew at 30°C, but growth was very poor at 37°C. DH10B/pS58 grew well at both temperatures. BRL3050/pRK248cI/pS63 grew very poorly at 30°C and not at all at 37°C, whereas DH10B/pRK248cI/pS63 grew well at 30°C, and poorly at 37°C. We interpreted these results to mean that the R·*Pvu*I endonuclease was more lethal to BRL3050 than to DH10B, especially at the higher temperatures when BRL3050 is most deficient in ligase. More extensive experimentation was not undertaken.

DISCUSSION

The R·*Pvu*I endonuclease and methylase genes are arranged in a tandem of the methylase gene followed closely by the endonuclease gene. These genes are followed by a terminator-like sequence immediately after the endonuclease gene. The M·*Pvu*I methylase did not completely protect the host DNA from digestion by R·*Pvu*I, even when it was cloned in a multicopy plasmid (pS59). Full protection was conferred by the methylase gene when it was cloned by PCR downstream from the lac promotor. The upstream region of the *Pvu*I operon, was not characterized, nor was the sequence of the region established unambiguously. The restriction map of the region was roughly in agreement with that published by Katsuragi, Kawakami, and Maekawa in patent applications (7, 8).

A striking result was that the endonuclease could be expressed without protection of the host by methylation. The level of production obtainable from unprotected *E.coli* (DH10B/pS58) was not small, and in fact was comparable to the amount obtained from the natural host, *Proteus vulgaris* (15,000 units/gram). While similar results have been reported for *Pae*R7I and *Taq*I (21, 22), many restriction enzymes, such as *Hha*II (10) are lethal to the host when expressed in the absence of methylase protection. How can a bacterium produce an enzyme that can cleave its chromosome into dozens of pieces? The major endonuclease of *E.coli* K12 (endonuclease I) is capable of destroying the host chromosome, but it does not because it is a periplasmic enzyme, compartmentalized away from the host chromosome (23). Additionally, endonuclease I is severely inhibited by RNA, which can be thought of as an intracellular inhibitor (23). The R·*Pvu*I endonuclease may not be as active intracellularly since *E.coli* is not the natural host for the R·*Pvu*I system, either because of inhibition by *E.coli* proteins of by potentiation by *P.vulgaris* proteins in the natural host. Since R·*Pvu*I does not seem to be periplasmic, the intriguing question remains: how is R·*Pvu*I prevented from degrading the chromosome, and why is this mechanism overcome when the enzyme is overproduced? It was reported that *Eco*RI endonuclease can cleave unmethylated host DNA in vivo, and that DNA ligase was necessary and perhaps sufficient to repair the damage (13). Our results confirm these observations, since a mutant deficient in ligase was more sensitive to R·*Pvu*I production. Since *E.coli* with normal levels of ligase can produce a moderate amount of R·*Pvu*I without methylase protection, one may even ask why the R·*Pvu*I restriction system requires the methylase at all, as long as the endonuclease is not overexpressed. Even though the laboratory strain DH10B/pS58 grew normally in the laboratory, the presence of the endonuclease without the corresponding methylase may well be deleterious to the survival of wild type strains in the environment. There must be some requirement for the methylase gene in the natural environment, since all restriction systems that have been tested are associated with corresponding modification system (1).

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