Volume 14 Number 20 1986 Nucleic Acids Research

Cloning the *Dde*I restriction-modification system using a two-step method

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Received 3 July 1986; Revised and Accepted 18 September 1986

ABSTRACT

*Dde*I, a Type II restriction-modification system from the gram-negative anaerobic bacterium *Desulfovibrio desulfuricans*, recognizes the sequence CTNAG. The system has been cloned into *E. coli* in two steps. First the methylase gene was cloned into pBR322 and a derivative expressing higher levels was constructed. Then the endonuclease gene was located by Southern blot analyses; *Bam*HI fragments large enough to contain the gene were cloned into pACYC184, introduced into a host containing the methylase gene, and screened for endonuclease activity. Both genes are stably maintained in *E. coli* on separate but compatible plasmids.

The *Dde*I methylase is shown to be a cytosine methylase. *Dde*I methylase clones decrease in viability as methylation activity increases in *E. coli* RR1 (our original cloning strain). Therefore the *Dde*I system has been cloned and maintained in ER1467, a new *E. coli* cloning strain engineered to accept cytosine methylases. Finally, it has been demonstrated that a very high level of methylation was necessary in the *Dde*I system for successful introduction of the active endonuclease gene into *E. coli*.

INTRODUCTION

Type II restriction-modification systems are being cloned with increasing rapidity. The first cloned systems were selected by phage restriction (*Hha*II, 1; *Pst*I, 2). But this method was frequently unsuccessful and often led to the selection of functions other than restriction genes. A large number of plasmid-borne systems have been readily transferred into *E. coli* cloning plasmids (*Eco*RI, 3, 4; *Eco*RII, 5; *Eco*RV, 6; *Pae*R7, 7, 8; *Pvu*II, 9). Finally, a growing number of systems are now being cloned by selection for an active methylase gene (*Bsu*RI, 10; *Taq*I, 11); since the two genes are often closely linked, both genes are cloned simultaneously. However, the methylase selection does not always yield a complete restriction system (*Bsp*RI, 12; *Msp*I, 13). Even attempts to clone larger regions adjacent to the methylase gene can fail to produce an active endonuclease gene.

In some systems the failure to clone a complete restriction-modification system may stem from trying to introduce the endonuclease gene into a host not adequately protected by methylation. If the methylase gene and endonuclease gene are introduced on a

common DNA fragment, the methylase must modify the host before the endonuclease cleaves the host genome. One solution would be to clone the genes in two steps: first introduce and overexpress the methylase gene and then clone the endonuclease on a compatible second vector. Previously cloned restriction-modification genes (*Eco*RI, *Hha*II) have been separated and introduced into *E. coli* on compatible plasmids (14, 15).

We describe here the cloning of the *Dde*I system from the gram-negative anaerobic bacterium *Desulfovibrio desulfuricans*. Its recognition and cleavage specificity is C↓TNAG (16, 17). In addition, we demonstrate that the *Dde*I system, when transferred to *E. coli*, acts as a classical restriction-modification system in that it can restrict unmodified phage.

MATERIALS AND METHODS

Bacterial Strains and Media

D. desulfuricans (NCIB8310) is an obligate anaerobe. 100 liters of cells were grown under anaerobic conditions in the following media: sodium lactate (6 g/liter); magnesium sulfate, heptahydrate (1 g/liter); sodium sulfate (2 g/liter); ammonium chloride (1 g/liter); potassium phosphate, dibasic (0.5 g/liter); calcium chloride, dihydrate (0.1 g/liter); yeast extract (1 g/liter); sodium chloride (25 g/liter); sodium thioglycollate (0.2 g/liter); ferrous sulfate (0.1 g/liter). After 2-5 days of growth at 30°C, cells were harvested by centrifugation and the pellet stored at -70°C.

E. coli strains HB101 and RR1 have been previously described (18). *E. coli* strain ER1467 (*mcr*B) is a derivative of strain JC1552 (19) containing a Tn10 insertion in *mcr*B (20). All *E. coli* strains were grown in LB Medium (21).

Plasmids and Phage

The plasmids pBR322 (22) and pACYC184 (23) were used for cloning. Plasmid DNA was prepared by the cleared lysate method (24) followed by CsCl/ethidium bromide ultracentrifugation (21) or by the alkaline lysis method (21).

The standard method of nomenclature previously described (7) was used to name plasmids carrying *Dde*I genes.

Phage stocks of λ_{vir} (New England Biolabs collection) and λ_{467}::Tn5 (gift of N. Kleckner via E. Raleigh) were prepared by plate lysates (21). All phage dilutions were done in SM solution (21).

Cloning Enzymes and Techniques

All restriction enzymes were produced at New England Biolabs and used according to recommended conditions. *Bam*HI-cleaved pACYC184 vector was treated with Calf Intestine Alkaline Phosphatase (Boehringer Mannheim) using conditions previously described (21); dephosphorylated *Hind*III-cleaved pBR322 was produced at New England Biolabs.

All ligations were done with 1000 units of T4 DNA Ligase (New England Biolabs) in a 100 μl reaction volume under prescribed conditions.

Transformations were done using the $CaCl_2$ heat shock method (25).

For Southern hybridizations, *E. coli* DNA Polymerase I (New England Biolabs), DNAse I (Sigma), and T4 Polynucleotide Kinase (New England Biolabs or Boehringer Mannheim) were used.

*Dde*I methylase clones were mapped using Tn5 mutagenesis (26) in HB101 (recA).

DNA Preparation

D. desulfuricans DNA was purified by the following method: 5 grams of frozen cells were resuspended in 20 ml of 25% sucrose, 50 mM Tris, pH 8.0. 10 ml of 0.25 M EDTA, pH 8.0 plus 6.0 ml of 10 mg/ml lysozyme in 0.25 M Tris, pH 8.0, was added. The suspension was left on ice for two hours. Then, 24 ml of 1% Triton X-100 (Rohm Haas), 50mM Tris, pH 8.0, 67 mM EDTA plus 5 ml 10% SDS were added to allow the cells to lyse. The lysed cells were phenol/chloroform extracted three times. The aqueous layer was transferred to dialysis tubing and dialyzed against four changes of 10 mM Tris, 1 mM EDTA, pH 8.0 over 24 hours. The dialyzed DNA solution was treated with RNase A (Sigma) to a final concentration of 100 μg/ml for one hour at 37°C. The solution was brought to 0.4 M NaCl, and 0.55 volumes of isopropanol were layered on top of the solution. The DNA was spooled with a glass rod, then dissolved in 15 ml of 10mM Tris, 1mM EDTA, pH 8.0. From 5 grams of cells, 1.5 mg of purified DNA was recovered.

DNA fragments from genomic and plasmid DNA restriction digests were gel purified as follows: 25-100 μg DNA cleaved with restriction enzymes was subjected to electrophoresis on a 1% agarose (SeaKem LE) gel in 0.5 μg/ml ethidium bromide and Tris-Acetate buffer (21). DNA was visualized under long-wave (375 nm) UV light. Appropriate fragments were excised from the gel and minced by extrusion through a 21.5 gauge needle, suspended in Tris-Acetate buffer, and subjected to ultracentrifugation at 290,000 x g in a type SW 50.1 rotor (Beckman) for 35 min at 4°C. The supernatant was brought to 0.4 M NaCl and the DNA was isopropanol precipitated. The DNA was resuspended, phenol extracted twice, ethanol precipitated, and resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0. A yield of approximately 75% was obtained.

A 5.0 kb *Mlu*I fragment from 100 μg of bacteriophage λ DNA was gel-purified using a Unidirectional Electroelutor (International Biotechnologies, Inc.) according to manufacturer's instructions.

Analysis of Methylase and Endonuclease Activity

To determine methylase activity *in vitro*, ^{3}H-methyl incorporation was measured as previously described (27) with the following modifications: 1 μg of λ DNA (New England Biolabs) was incubated with 5 μl of crude extract (7) for 30 min. at 37°C. A determination of *in vivo* methylase activity was made by passage of λ_{vir} phage through

the test host, using the method previously reported (7) substituting λ_{vir} for ϕ80 phage and *Dde*I endonuclease for *Pae*R7 (Fig. 2).

In vitro endonuclease activity was determined as described previously (27) except 1 μg of *Eco*RI linearized pBR322 was the substrate. Phage restriction tests were done using λ_{vir} to measure *in vivo* endonuclease activity, as previously described (7).

*Dde*I Endonuclease Purification

The 23 gram frozen pellet was thawed on ice and resuspended in 120 ml of Sonication Buffer (10 mM KPO_4, pH 6.9, 10 mM β-mercaptoethanol, 0.1 mM EDTA), 50 mM NaCl. All steps described below were performed at 4°C. Lysozyme was added to a final concentration of 300 μg/ml, and cells were ruptured by sonication. The cell lysate was centrifuged at 10,000 x g for 45 min.

The 120 ml supernatant was loaded on a 2.5 x 15 cm phosphocellulose P11 (Whatman) column equilibrated with Sonication Buffer, 150 mM NaCl. The enzyme was eluted with a linear gradient of 700 ml, from 150 mM to 1 M NaCl. The endonuclease eluted at approximately 0.5 M NaCl. Peak fractions were collected and dialyzed against Sonication Buffer, 50 mM NaCl.

The 170 ml enzyme pool from the phosphocellulose column was loaded on a 1.5 x 15 cm Heparin Sepharose (Pharmacia) column equilibrated with Sonication Buffer, 25 mM NaCl. A 340 ml gradient from 25 mM to 1M NaCl was used, and the enzyme eluted at approximately 0.5 M NaCl. Fractions with endonuclease activity were pooled and dialyzed against HPLC Buffer (20 mM Tris, pH 7.5, 10 mM β-mercaptoethanol), 50 mM KCl.

The 10 ml enzyme pool was passed through a Mono Q column (Pharmacia) and a Polyanion SI column (Pharmacia), then absorbed to a 1 x 8 cm Mono S column (Pharmacia) at 50 mM KCl. A 53 ml linear gradient was developed from 50 mM to 1 M KCl. The endonuclease eluted as a single peak at approximately 0.2 M KCl.

A Waters Associates Liquid Chromatograph was used to prepare the sample for protein sequencing in the following manner. *Dde*I endonuclease samples (15 μg) were subjected to chromatography on a Vydac C4 214TP54 (5 μm, 4.6 X 300 mm) 300 Å pore reverse phase column, developed with a linear gradient of 5% to 50% acetonitrile in 0.1% trifluoroacetic acid over 25 minutes at a flow rate of 1 ml/min with detection at 214 nm. Individual peaks were collected manually and lyophilized.

Protein Sequencing and Synthesis of the Endonuclease Probe

The sequential degradation of proteins was performed with an Applied Biosystems model 470A gas-phase sequenator (28). The first 19 phenylthlohydantoins were unambiguously identified by high-performance liquid chromatography on an IBM Cyano (5 μm, 4.5 X 250 mm) column with slight gradient modifications from those previously described (29).

The protein sequence was used to derive a DNA sequence with minimum degeneracy. This tetradecamer was synthesized using a DNA Synthesis Kit (New England Biolabs) and purified by chromatography on a Water Associates C8 (10 μm, 0.5 x 10 cm) Radial Pak column.

Southern Hybridizations

1-2 μg of cleaved genomic DNA or 0.5-1 μg of digested plasmid DNA was subjected to agarose gel electrophoresis and transferred to nitrocellulose (Schleicher and Schuell) by the method of Southern (30).

The methylase probe was prepared by gel-purifying the 3 kb *Hind*III fragment from pDdeM3.0a. One μg of the fragment was nick-translated (31) using 100 pmole [α-^{32}P]dATP (New England Nuclear, 800 C_i/mmol). Hybridizations were performed as previously described (21) at 65°C; washes were carried out at 55°C.

For the oligonucleotide probe of the endonuclease gene, 100 ng of the probe was kinased (32) using γ-^{32}P-ATP (New England Nuclear, 3000 C_i/mmol). Hybridizations were performed by a method previously described (33, 34) at room temperature, followed by filter washes at 37°C.

RESULTS

Cloning the Methylase Gene and Characterization of Its Activity

The *D. desulfuricans* library was made by incubating 10 μg *D. desulfuricans* DNA with 15 units of *Hind*III in a 100 μl reaction for 1 hour. After heat denaturing the enzyme, the digested DNA was ligated to *Hind*III-cleaved and dephosphorylated pBR322 in an insert to vector ratio of 2:1. This ligation mix was used to transform competent RR1 cells, and transformants were selected for ampicillin resistance. A mixed population of approximately 10^5 transformants were suspended in 10 mM Tris, pH 7.5, 10 mM $MgCl_2$ buffer. The pooled transformants were used to inoculate 500 ml of LB, and grown to saturation, then plasmids were isolated and CsCl-ethidium bromide purified. A yield of approximately 100 μg of DNA was obtained.

*Dde*I methylase clones were selected for their resistance to cleavage by *Dde*I endonuclease. 5 μg of recombinant plasmids were overdigested with 80 units of *Dde*I endonuclease for 1 hour, transformed into $CaCl_2$-treated RR1 cells, and selected for ampicillin resistance. A comparison of endonuclease-challenged and unchallenged recombinant plasmids revealed a 10^3 selection. Transformants were tested for resistance to *Dde*I endonuclease, and digested with *Hind*III. Two types of recombinants resistant to *Dde*I were isolated, carrying a 3.0 kb *Hind*III fragment in opposite orientations. These were designated pDdeM3.0a and pDdeM3.0b. Crude extracts of these clones did not show any *Dde*I endonuclease activity.

The position of the methylase gene within the 3.0 kb *Hind*III fragment was deter-

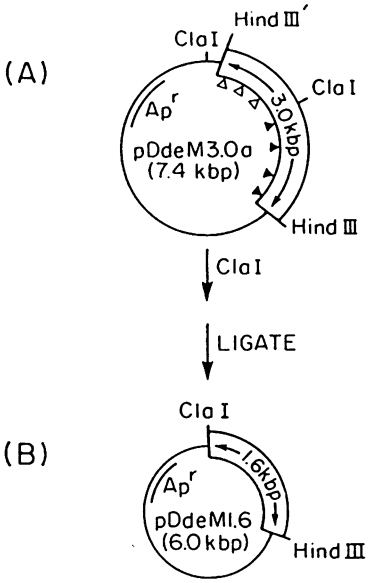

Fig. 1. Tn5 Mapping of pDdeM3.0a and Construction of pDdeM1.6. (*A*) pDdeM3.0a methylase clone carrying 3.0 kb *Hind*III insert. pDdeM3.0a and pDdeM3.0b clones are identical except the insert is present in the opposite orientations relative to pBR322. Triangles (▼, ▽) represent sites where Tn5 inserted, determined by digesting with *Hind*III. Inactivation of the M.*Dde*I gene by Tn5 was tested by digestion with R.*Dde*I, and is symbolized as a solid triangle (▼). Tn5 insertions that do not cause methylase inactivation are represented by an open triangle (▽). (*B*) pDdeM1.6 was constructed by digesting pDdeM3.0a with *Cla*I. Following heat inactivation of the enzyme, the digested plasmid was self-ligated at a concentration of 1 μg/ml. Ampicillin resistant transformants were selected and screened for loss of the *Cla*I fragment.

mined by a series of Tn5 mutagenesis experiments (Fig. 1A). Only insertions between *Cla*I and *Hind*III sites resulted in the loss of *Dde*I methylase activity, suggesting that the gene was contained in the region between these sites. By deleting the *Cla*I fragment from the pDdeM3.0a, a subclone designated pDdeM1.6 was generated that maintained methylase activity, confirming that the methylase gene lay entirely within this 1.6 kb region (Fig. 1B).

Methylase activity of the three M.*Dde*I clones was compared using an *in vivo* λ protection assay and an *in vitro* ^{3}H-methyl incorporation assay. Results of the λ protection assays are shown in Fig. 2. Both assays revealed a gradation of methylase activity, with pDdeM3.0b having the lowest activity and pDdeM1.6 having the highest; only pDdeM1.6 has enough activity to completely protect λ phage *in vivo*. *In vitro* assays

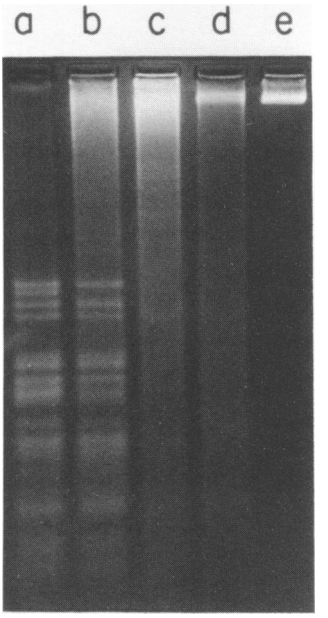

Fig. 2. *In Vivo* λ Protection Assay. λ DNA was modified in ER1467 carrying the following methylase plasmids: lane (b): pDdeM3.0b; (c): pDdeM3.0a; (d): pDdeM1.6; then challenged with R.*Dde*I. Controls were done using unmodified, purified λ DNA: lane (a): *Dde*I-cut λ DNA; (e): uncut λ DNA. ER1467 cells containing M.*Dde*I plasmids were grown to mid-log then infected (moi=10^{-1}) with phage λ. DNA was prepared as previously described (7). Phage DNA was incubated with 15-fold excess *Dde*I and subjected to electrophoresis on a 1.8% agarose gel.

were consistent with these results (data not shown). pDdeM1.6 was therefore used in later attempts to clone the endonuclease gene (see below).

It is possible to demonstrate that M.*Dde*I is a cytosine rather than an adenine methylase in the following way: λ DNA contains overlapping *Sau*3AI (GATC) and *Dde*I (CTNAG) sites at position 2532: 5'-GATCTCAG-3' (34, 35). Cleavage of λ DNA with *Sau*3AI results in 165 bp and 487 bp fragments from this region. A 5.0 kb *Mlu*I fragment contains the overlapping *Sau*3AI and *Dde*I sites. This fragment was isolated so that the relevant *Sau*3AI fragments could be better visualized. We treated this λ fragment with M.*Dde*I and then challenged with *Sau*3AI. If M.*Dde*I were an adenine methylase, the modification would occur on the second adenine within this sequence, outside the *Sau*3AI recognition site. *Sau*3AI cleavage would therefore result in the normal 165 bp and 487 bp fragments. If, however, M.*Dde*I is a cytosine methylase, modification would occur on the cytosine within the *Sau*3AI recognition sequence. Hemi-methylation

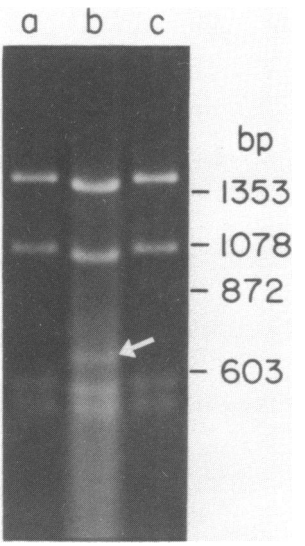

Fig. 3. M.*Dde*I is a Cytosine Methylase. Lanes (a) and (c): unmodified λ/*Mlu*I fragment cut with *Sau*3AI; lane (b): M.*Dde*I-modified λ/*Mlu*I fragment cut with *Sau*3AI. To modify the purified λ/*Mlu*I fragment, the DNA was incubated with 1 μl of M.*Dde*I crude extract (from pDdeM1.6) then challenged with *Sau*3AI endonuclease. Arrow shows the appearance of a 652 bp band that is absent in the unmethylated control.

within the *Sau*3AI site blocks *Sau*3AI cleavage; only single-strandod nicking occurs (36). *Sau*3AI treatment would then result in the appearance of a new 652 bp fragment. As shown in Fig. 3, the new band does in fact appear, indicating that M.*Dde*I is a cytosine methylase.

Realization that M.*Dde*I is a cytosine methylase helped explain the following phenomenon. The clone that produces the most methylase, which carries pDdeM1.6, was considerably less viable than pDdeM3.0a and pDdeM3.0b constructs in our cloning strain RR1. When a culture of RR1 carrying pDdeM1.6 reached stationary phase, it had only 6% the number of viable cells as did a parallel culture of RR1 alone (Table I). It has been recently shown that certain *E. coli* strains have systems that block introduction of DNA containing cytosine methylation (20); the locus responsible for this activity has been designated '*mcr*B' (modified cytosine restriction, 20). In RR1, this locus is derived from *E. coli* B and is of different specificity and apparently weaker activity (20, 37). A new series of cloning strains has been constructed by inactivating this locus with Tn10 insertions. We introduced our methylase clones into one of these strains, ER1467: comparison of pDdeM3.0a, pDdeM3.0b, pDdeM1.6, and ER1467 alone shows a complete restoration of viability; there was a negligible difference in the number

TABLE I. VIABILITY OF METHYLASE CLONES IN RR1 vs. ER1467

HOST	M.*Dde*I PLASMID	C.F.U./ml	VIABILITY[1]
RR1	————	4.8×10^8	1.00
RR1	pDdeM3.0b	4.1×10^8	0.85
RR1	pDdeM3.0a	2.7×10^8	0.56
RR1	pDdeM1.6	0.3×10^8	0.06
ER1467	————	6.3×10^8	1.00
ER1467	pDdeM3.0b	7.2×10^8	1.14
ER1467	pDdeM3.0a	7.0×10^8	1.11
ER1467	pDdeM1.6	5.7×10^8	0.90

[1] Viability is defined as the ratio of c.f.u./ml of the test strain to the c.f.u./ml of the host (RR1 or ER1467) alone at saturation.

of colony-forming units (c.f.u.) at saturation (Table I). Therefore, further cloning and characterization of the *Dde*I system were done in ER1467.

Cloning the Endonuclease Gene

Since endonuclease and methylase genes are frequently closely linked, it seemed reasonable to expect that the R.Dde I gene would lie in a region contiguous with the methylase gene. The region around the methylase gene on the *D. desulfuricans* genome was mapped by Southern hybridization analysis using the 3.0 kb *Hind*III fragment from pDdeM3.0a as a probe. Results are shown in Fig. 4A.

Assuming that the endonuclease gene is in proximity, we wished to determine on which side of the methylase gene it lay. Having homogeneous protein, we were able to make an oligonucleotide probe specific for the 5'-terminus of the endonuclease gene. The *Dde*I endonuclease ran as a single band of 30,000 daltons on an SDS-acrylamide gel (data not shown), and has a specific activity of 10^5 units/mg, as measured on λ DNA. The first 19 amino acids of the endonuclease are Met-Lys-Ala-Ala-Thr -Asp-Gln-Glu-Leu-Arg-Lys-Leu-Ile-Val-Leu-Tyr-Asn-Asn-Val. Using the five amino acids underlined, a mixed tetradecameric probe was prepared with the sequence: 5'-ATG-AAR-GCN-GCN-AC-3' (R, nucleotide A or G; N, nucleotide A,G,C, or T). Southern blots of genomic DNA digests were hybridized with the probe (data not shown). The endonuclease probe does not hybridize to the 3.0 kb *Hind*III fragment known to contain the methylase gene.

The Southern blots were used to generate the restriction map represented in Fig. 4B. Two enzyme digests yielded fragments that hybridized to both probes and that were large enough to code for the methylase and/or the endonuclease. Both probes hybridize to a single *Pst*I fragment as shown in lane (a), Fig. 4A, suggesting that both

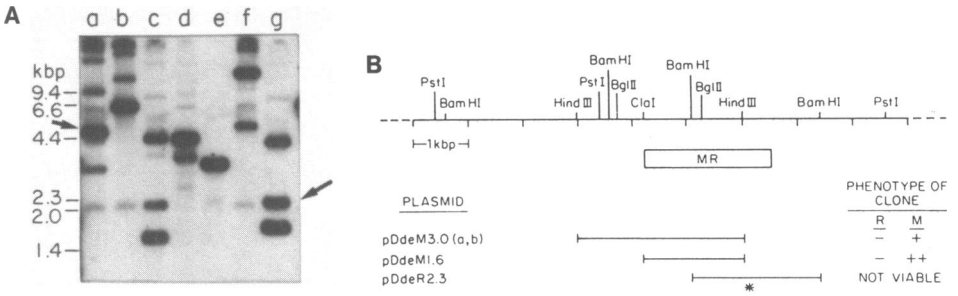

Fig. 4. Southern Blot Analysis and Restriction Map of *Dde*I Clones. (*A*) Southern blot using 3.0 kb *Hind*III insert from pDdeM3.0a to probe genomic DNA digests. Enzymes used: (a) *Pst*I; (b) *Sal*I; (c) *Bgl*II; (d) *Bcl*I; (e) *Hind*III; (f) *Eco*RI; (g) *Bam*HI. Arrows in lanes (a) and (g) indicate bands which also hybridize to the oligonucleotide probe specific for the 5'-terminus of the endonuclease gene. (*B*) Restriction Map of *D. desulfuricans* Genome in the Region Containing the *Dde*I System and Derivative Plasmids. 'M R' represents the positions of the methylase and endonuclease genes, respectively. pDdeM3.0a, pDdeM3.0b and pDdeM1.6 are pBR322 derivatives carrying the methylase gene; pDdeR2.3 is a pACYC184 derivative carrying the endonuclease gene alone. The star (—*—) represents the fragment that hybridizes to the oligonucleotide probe.

the endonuclease and the methylase genes are located on this 4.8 kb piece of DNA. In addition, both probes hybridize to a 2.3 kb *Bam*HI fragment, as shown in lane (g), Fig. 4A. This fragment includes a portion of the methylase gene and is large enough to encode the entire endonuclease gene.

Attempts to clone the 4.8 kb *Pst*I fragment were unsuccessful (see next section). Therefore the endonuclease and methylase genes were introduced separately on compatible plasmids in the following manner. 2.3 kb *Bam*HI fragments of *D. desulfuricans* DNA were cloned into pACYC184: *D. desulfuricans* DNA was cut with *Bam*HI and fragments in the appropriate size range were gel purified. These fragments were ligated to *Bam*HI-cut and dephosphorylated pACYC184 and used to transform ER1467 cells carrying the M.*Dde*I clone pDdeM1.6. Transformants were selected for ampicillin and chloramphenicol resistance. Fifty transformants were picked into microtiter wells (Nunc) and replica-plated on six plates seeded with λ_{vir} phage dilutions from 10^4-10^8 phage/plate. A single transformant restricted phage at a level of approximately 10^{-1}. A crude extract of this transformant, designated pDdeR2.3, confirmed the presence of active *Dde*I endonuclease. DNA from this clone was prepared, and preliminary restriction mapping demonstrated that this 2.3 kb *Bam*HI fragment overlapped the *Bam*HI to *Hind*III portion of pDdeM1.6 as shown in Fig. 4B. Thus the *Dde*I system is contained within 2.9 kb of *D. desulfuricans* DNA.

Level of Methylation Required for Cloning the Endonuclease

All previously cloned restriction-modification systems were moved into *E. coli* on a single DNA fragment, but attempts to clone the *Dde*I system in this way were unsuccessful. Southern blot analysis showed that a 4.8 kb *Pst*I fragment carried the complete *Dde*I restriction-modification system, but a methylase selection of a *Pst*I-cut *D. desulfuricans* library did not produce any clones. In contrast, the *Hind*III-cut library produced methylase clones, although the 3.0 kb *Hind*III insert did not include the endonuclease. That the endonuclease gene was finally cloned in two steps using pDdeM1.6, which expresses a high level of methylase, suggested that the level of methylation may be critical.

We had noticed that the original transformants pDdeM3.0a and pDdeM3.0b did not completely methylate either their own plasmid DNA or infecting λ DNA; only the overexpressing construct pDdeM1.6 was able to do this. We therefore tested the possibility that undermethylated transformants would be unable to support an active endonuclease gene. The endonuclease clone was introduced into cells containing the pDdeM3.0a, pDdeM3.0b, or pDdeM1.6 plasmid. Ampicillin, chloramphenicol resistant transformants were selected and tested for their ability to restrict λ_{vir}. The results were striking: all transformants containing pDdeM1.6 (the 'hypermethylator') restricted λ_{vir}. In contrast, none of the transformants containing pDdeM3.0a or pDdeM3.0b restricted λ_{vir}. When plasmid DNA from these transformants was analyzed, it was shown that the endonuclease gene had been interrupted; preliminary restriction mapping suggested that IS elements inserted into the gene.

DISCUSSION

When cloning restriction-modification systems, one must consider expression of both genes and their effect upon the host. In the absence of sufficient methylase activity, the endonuclease can be lethal to the cell, though in some cases cells have been found to tolerate a low level of endonuclease activity in the absence of methylase (7). In the *Dde*I system, the endonuclease gene was inactivated in the presence of low levels of methylase, but remained active when higher levels of methylase were present in the cell. Thus, insufficient levels of methylase activity may be one reason why certain endonucleases are difficult to clone.

Another potential difficulty in cloning restriction-modification systems also affected the *Dde*I system. This involves the pronounced incompatibility between certain cytosine methylases and the *mcr* genes present in most strains of *E. coli* (9, 20). To avoid inactivation of the endonuclease gene, methylase expression had to be increased to a sufficient protective level. Although low levels of cytosine methylation were tolerated in

the original host, RR1, higher levels of methylase activity were not. RR1 carries *mcr*B information from *E. coli* B. This *mcr*B function is of different specificity than the K12 activity (20, 37) and may have a weaker effect. Therefore we worked in an *mcr*B::Tn10 host ER1467. These two approaches, taken together, may greatly facilitate the isolation of restriction-modification genes hitherto unclonable.

We are continuing characterization the the *Dde*I system by determining its DNA base sequence. It is unclear why the *Dde*I methylase gene is so much better expressed in pDdeM1.6 than on its parent plasmids, pDdeM3.0a and pDdeM3.0b; DNA sequence analysis now in progress might assist in understanding this phenomenon.

Finally, the *Dde*I oligonucleotide has proved to be very useful. The probe was first successfully employed to locate the *Dde*I endonuclease on the genome. We are currently using it in conjuction with the DNA sequence analysis to position and orient the endonuclease gene on the pDdeR2.3 plasmid.

ACKNOWLEDGEMENTS

We thank Drs. R. Blumenthal, A. Bhagwat, A. Kiss, T. Gingeras, R. Roberts, I. Schildkraut and D. Comb for helpful criticism of the manuscript. We would also like to especially thank Dr. Lisa Raleigh for stimulating discussions and provision of cloning strains.

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