Two new restriction endonucleases from Proteus vulgaris


Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, and New England Biolabs, Beverly, MA 01915, USA

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ABSTRACT

Two novel sequence-specific endonucleases have been isolated from Proteus vulgaris, ATCC 13315. Pvul recognizes the sequence:

5' C G A + T + C G 3'
3' G C + T A G C 5'

and PvulI recognizes the sequence:

5' C A G + C T G 3'
3' G T C + G A C 5'

and cleave as indicated by the arrow (+).

Pvul is an isoschizomer of XorII, RshI, and XmI. No enzyme with the specificity of PvulI has been described previously.

INTRODUCTION

Several members of the family Enterobacteriaceae have been shown to possess Type II restriction endonucleases, e.g., Escherichia coli (EcoRI), Klebsiella pneumoniae (KpnI), Enterobacter cloacae (EclII), Serratia marcescens (SmaI), Providencia stuartii (PstI) and Salmonella infantis (SmiI) (1 and references therein). We have examined a number of strains from the genus Proteus for the presence of Type II restriction endonucleases and have found one species, Proteus vulgaris, ATCC 13315, which contains two such enzymes. We now describe the isolation and characterization of two specific endonucleases, Pvul and PvulI, from this strain.

Isolation of Restriction Endonucleases

1. Growth of Cells. P. vulgaris (ATCC 13315) was grown on nutrient broth (Difco) at 37°C and harvested during stationary phase. The yield of cells was 2-3 grams/liter. Cells (10 gm) were suspended in 0.01 M Tris-HCl, pH 7.5, 0.01 M SHCH2CH2OH (17ml) and sonicated (10 x 30 sec).
Following centrifugation at 100,000 g for 90 min, the supernatant was adjusted to 1 M NaCl and applied to a Biogel A 0.5 M column (100 cm x 2.5 cm diameter) which had been previously equilibrated with 1.0 M NaCl, 0.01 M Tris-HCl, pH 7.5, 0.01 M SHCH₂CH₂OH. The assay of this column is shown in Figure 1. PvuI elutes first from this column very near to the void volume. Peak fractions of PvuI (24-36) were combined, omitting as much as possible of the nonspecific nuclease which elutes just beyond the peak fractions, and dialyzed against a buffer containing 10% glycerol, 0.01 M potassium phosphate pH 7.4, 0.01 M SHCH₂CH₂OH and 10⁻⁴ M Na₂EDTA (PC buffer). It was then applied to a DEAE cellulose column (Whatman DE-52) (15 x 2.0 cm diameter) which had been previously equilibrated.

Figure 1. Assay of fractions from the Biogel A 0.5m column
Samples (3 µl) of column fractions (4.2 ml) were incubated at 37°C for 3 hrs in a reaction mixture (50 µl) containing 2 µg of λ DNA, 6 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 6 mM SHCH₂CH₂OH. After digestion, 10 µl of a solution containing 50% (weight/volume) sucrose, 0.2% (w/v) bromophenol blue were added in the mixture and loaded onto a 1.4% (w/v) agarose slab gel, 20 cm x 20 cm x 0.3 cm. Electrophoresis was carried out at 150 volts for 3 hrs. Bands were stained with ethidium bromide, and photographed during ultraviolet light irradiation. Fraction numbers are indicated above each channel.
with PC buffer. After washing with PC buffer, the column was eluted with a linear gradient (200 ml total volume) from 0 to 1.0 M KCl in PC buffer. The assay of this column is shown in Figure 2.A. PvuI elutes from this column both during the wash and around 0.5 M KCl. The latter fractions were heavily contaminated with a nonspecific nuclease and comprised the smaller part of the total activity. The enzyme in the wash (fractions 26-36) was applied to a phosphocellulose column (Whatman P-11) (25 x 1.2 cm diameter) that had been previously equilibrated with PC buffer. Elution was with 200 ml of a linear gradient from 0 to 1.0 M KCl in PC buffer. PvuI elutes between 0.48 and 0.6 M KCl. Peak fractions from this column were combined, dialyzed against PC buffer, and then applied to a second DEAE cellulose column (25 x 1.2 cm diameter). Elution was with 200 ml of a linear gradient from 0 to 1.0 M KCl in PC buffer. PvuI elutes between 0.4 to 0.5 M KCl. Peak fractions were combined, dialyzed against PC buffer containing 50% glycerol. Optimal activity of the purified PvuI enzyme was found to occur when the final digest conditions include 150 mM NaCl, 100 mg/ml of bovine serum albumin. 1400 units of PvuI were isolated from 10 gm wet weight of cells. 1 unit of PvuI is defined as the amount of enzyme which completely digests 1 ug of Ad2 DNA in 1 hr at 37°C. Although digestion of 2 ug of Ad2 DNA for 18 hr with 15 units of this enzyme preparation gave sharp bands on agarose gels, trace amounts of a contaminating nuclease were still present (see text).

**Isolation of PvuII.** PvuII elutes from the Biogel column later than PvuI and immediately following a peak of nonspecific nuclease. Peak fractions (78-90) were combined, omitting as much as possible of the nonspecific nuclease, and dialyzed against PC buffer. Combined fractions were dialyzed against PC buffer and applied to a DEAE cellulose column (15 x 2.0 cm diameter) which had previously been equilibrated with PC buffer. Elution was with 200 ml of a linear gradient from 0 to 1.0 M KCl in PC buffer. PvuII elutes between 0.4 and 0.5 M KCl. Peak fractions were combined, dialyzed against PC buffer, and applied to a phosphocellulose column (25 x 2.0 cm diameter) which had been previously equilibrated with PC buffer. Elution was with 400 ml of a linear gradient from 0 to 1.0 M KCl. The assay of this column is shown in Figure 2b. PvuII elutes at approximately 0.7 M KCl. Peak fractions were pooled, dialyzed against PC buffer and rechromatographed on
phosphocellulose (15 x 1.2 cm diameter). Elution was with 200 ml of a linear gradient from 0.4 to 1.5 M KCl. PvuII elutes between 0.65 and 0.7 M KCl. Peak fractions were combined and concentrated by dialysis against PC buffer containing 50% glycerol. The yield of PvuII from 10 g cells was about 3,700 units. 1 unit of PvuII is defined as the amount of enzyme required to completely digest 1 μg of Ad2 DNA in 1 hr at 37°C.

RESULTS AND DISCUSSION

Six strains from the genus Proteus were initially screened for the presence of type II restriction endonucleases. These included P. mirabilis (ATCC 4675) and five strains of P. vulgaris (ATCC 6059; 9920; 12454; 13315; 21117). In each case, about 10 gms of cells were grown and an S-100 prepared by sonication and centrifugation. This extract was then fractionated on Biogel A 0.5 M and fractions assayed for the presence of a specific endonuclease using both bacteriophage lambda and adenovirus-2 DNAs as substrate. Only in the case of P. vulgaris, ATCC 13315, could we detect specific endonucleolytic activity. Two distinct peaks of activity were visible upon assay of the Biogel column (Figure 1) and these were separated and purified as described in Methods. Each enzyme was characterized and its recognition sequence determined.

Characterization of PvuI

PvuI was initially characterized by its action on various DNAs (Figure 3). Adenovirus-2 DNA is cleaved 6 times, lambda DNA 3 times, while SV40 DNA and ϕX174 DNA are resistant to cleavage. Our initial attempts to determine the recognition sequence involved terminal labeling with polynucleotide kinase of PvuI fragments of Ad2 DNA and analysis of the 5' terminal nucleotide by standard procedures (2). For several different preparations of the enzyme, this method gave ambiguous
Figure 3. Agarose gel electrophoresis of PvuI and PvuII digests

DNA samples (0.5 to 2.0 μg) were incubated with 1 to 5 units of the indicated endonuclease for 2 hrs at 37°C and fractionated on a 1.2% Agarose slab gel, as described in the legend to Figure 1. Slot 1, PvuI on Ad2 DNA; Slot 2, PvuI + MboI on Ad2 DNA; Slot 3, MboI on Ad2 DNA; Slot 4, PvuI on Ad2 DNA; Slot 5, PvuI on λ DNA; Slot 9, PvuI + AluI on Adenovirus DNA; Slot 10, AluI on Ad2 DNA; Slot 11, PvuII on Ad2 DNA; Slot 12, PvuII on λ DNA; Slot 13, PvuII on SV40 DNA; Slot 14, PvuII on φX 174 RF DNA. The DNAs in channels 6, 7, and 14 contain supercoiled, relaxed circular and non-specific linear forms.

results with no clearly defined terminal nucleotide. We next used a computer program (3) to analyze the cleavage patterns produced on 6 different sequence DNA substrates and this led to a predicted recognition site CGATCG. Preliminary indication that this might be correct was obtained by recognizing that the central tetranucleotide of this sequence is equivalent to the MboI recognition site (5' GATC 3') (4). A digest of Ad2 DNA or lambda DNA with either MboI alone or a mixture of PvuI and MboI should give identical digestion patterns. Figure 3 demonstrates that this is so. Our failure to observe a unique 5' terminal nucleotide in our earlier experiments could thus be interpreted to mean that cleavage was taking place outside of the recognition sequence or, alternatively, that our enzyme preparation was contaminated.
with another nuclease which was either introducing random nicks or was sequentially degrading the 5' terminus. When analyzing fragments produced by several different preparations of Pvul we observed that pC was the most intensely labeled of the 5' terminal nucleotides and pG was the second most intensely labeled. However, even in our best experiment, only 44% of the total label was present in pC. In another experiment in which the 3' end of Pvul fragments of Ad2 DNA were labeled using terminal transferase, we found that dTp represented more than 60% of the labeled nucleotides. From these results, we conclude that our preparations of Pvul contain trace amounts of a 5' → 3' exonuclease.

Because of these inherent difficulties, we used a second method (5, 6) to unambiguously identify the recognition sequence and cleavage site of Pvul. An M13 clone containing a fragment of bacteriophage lambda DNA was found to contain a single Pvul site within the insert. Template DNA was prepared from this clone and used for chain elongation with the Klenow fragment of E. coli DNA polymerase I. Following elongation, the polymerase was inactivated by heat treatment and the extended chains cleaved with Pvul. To one-half of the reaction a further amount of DNA polymerase I, plus all four deoxynucleoside triphosphates, were added and the reaction continued. To the other half no addition was made. These two samples were then electrophoresed on a DNA sequencing gel alongside a standard set of sequencing reactions (7). The results are shown in Figure 4. From this experiment, it can be seen that cleavage does indeed take place within the sequence CGATCG, as predicted from our earlier analysis, and furthermore that the site of cleavage lies between the T and C residue on both strands. In the case of the newly-synthesized strand, it can be seen that the band obtained in the absence of added polymerase comigrates with the band in the T channel, indicating that T is a 3' terminal nucleotide, whereas in the reaction in which polymerase has been added, a band two nucleotides shorter is produced due to resection of the 3' terminal dinucleotide by the 3' → 5' exonuclease of DNA polymerase I. These data firmly establish that the recognition site for Pvul is CGATCG with the site of cleavage lying as indicated by the arrow.

Cleavage site of XorII

Like Pvul, XorII has been reported (9) to recognize the sequence CGATCG, but to cut at a different location within that sequence, producing fragments with a tetranucleotide 3'-extension. Because
Figure 4. Recognition and Cleavage Site of PvuI

A recombinant M13 phage composed of a fragment of bacteriophage λ DNA, containing a PvuI site, inserted into the vector mp7 (14) was used as template and a synthetic oligonucleotide complementary to the vector was used as primer. The sequence channels were obtained by the chain-termination procedure (7). Channels 1 and 2 were obtained from a parallel reaction in which the primer was extended in the absence of chain terminators and the reaction mixture was treated with PvuI following elongation. One-half of this mixture was loaded in channel 1. The other half was further treated with DNA-polymerase I and loaded in channel 2.

rather few examples are known of enzymes which recognize an identical sequence and cleave at different locations within it, we have re-examined the site of cleavage by XarII. This was done by preparing 5' terminally-labeled XarII fragments of Ad2 DNA and analyzing the 5' terminal nucleotides produced upon complete digestion with pancreatic DNase and venom phosphodiesterase. We find that the predominant 5' terminal mononucleotide produced is pC. Taken together with the published results (9), this would suggest that XarII cleaves the CGATCG recognition sequence in a manner similar to PvuI to generate a 3'-dinucleotide
The previous assignment for the site of cleavage by XorII, between the C and G residue within the recognition sequence, was based upon an experimental strategy rather similar to the one that we have used for the determination of the Pvul recognition sequence. An important difference is that the sequencing standard in that case was prepared using the Maxam-Gilbert procedure (11). This means that the chemical nature of the bands present in the sequence standard differed from those present in the cleaved samples in that the latter carry an additional 3'-phosphate. Consequently, the bands may not exactly line up on the sequencing gel and this potential anomaly must be taken into account when interpreting the results. The extent of the anomaly increases dramatically with decreasing chain length and we believe that it is this factor which led to the erroneous interpretation of the previous results with XorII.

Determination of Recognition Site for PvuII

PvuII was initially characterized by its action on Ad2 DNA (>22 sites), lambda DNA (15 sites), SV40 DNA (3 sites) and 0X174 DNA (no sites) (Figure 3). The recognition sequence for PvuII was determined in the usual way (2), by preparing PvuII fragments of Ad2 DNA and labeling them at their 5' ends with polynucleotide kinase and γ<sup>32</sup>P-ATP. The 5' terminal nucleotide released after digestion of these labeled fragments with a mixture of pancreatic DNase and snake venom phosphodiesterase was predominantly pC, while the 5' terminal dinucleotide obtained after digestion with exonuclease I was pCT. A two-dimensional analysis of the oligonucleotides produced by digestion with pancreatic DNase alone are shown in Figure 5. From this experiment it can be seen that a unique sequence, pCTG, is present on all fragments but that this sequence then becomes degenerate at the fourth nucleotide. The simplest interpretation of these results is that PvuII recognizes the hexanucleotide sequence CAG+CTG with the site of cleavage indicated by the arrow. The central tetranucleotide of this sequence, AGCT, is also the recognition site for the enzyme AluI from Arthrobacter luteus (8) and double digests between PvuII and AluI show no additional bands when compared with digests by AluI alone (Figure 3).

Conclusions

Two enzymes have previously been reported which have the same recognition sequence as Pvul. These are the enzymes XorII from
Ad2 DNA (10 μg) was incubated in a reaction mixture (100 μl) containing 6 mM Tris-HCl (pH 7.9), 6 mM MgCl₂, 6 mM S-butyrylglucose, 10 units PvuII for 1 hr at 37°C. Following extraction with phenol (2 x 200 μl) the DNA fragments were precipitated with ethanol and recovered by centrifugation. The fragments were resuspended in 50 μl of solution containing 100 mM Tris-HCl (pH 7.9), 10 mM MgCl₂, 2 μg of alkaline phosphatase and incubated for 30 min at 37°C. Following extraction with phenol (2 x 100 μl) the dephosphorylated DNA fragments were precipitated with ethanol and recovered by centrifugation. The DNA was phosphorylated in a reaction mixture (100 μl) containing 50 mM Tris-HCl (pH 9.5), 10 mM MgCl₂, 5 mM dithiothreitol, 5% glycerol, 10 mM γ-[³²P]-ATP (activity 1 Ci/mM), 10 units of polynucleotide kinase and incubated for 1 hr at 37°C. After adjusting this solution to 0.3 M sodium acetate the DNA fragments were precipitated with 3 volumes of ethanol. The precipitate was dissolved in 100 μl of 10 mM Tris-HCl (pH 7.9) and unreacted γ-[³²P]-ATP was removed by passage through a Sephadex G50 column run in 1 mM
Tris-HCl (pH 7.9) 1 mM Na₂ EDTA. Labelled PvuII fragments were recovered from the void volume by precipitation with ethanol (2 volumes) and centrifugation. The DNA was incubated in a reaction mixture (5 μl) containing 0.1 M Sodium acetate (pH 5.0), 5 mM MgCl₂ and 2 μg of pancreatic DNase for 30 min at 37°C, and fingerprinted using electrophoresis on cellulose acetate at pH 3.5 in the presence of 7 M urea for the first dimension and homochromatography (12) on a thin layer of DEAE-cellulose (1:10) eluting with homomix-6 (13) for the second dimension. The 5’-oligonucleotides were detected by autoradiography.

Xanthomonas oryzae (9) and RshI from Rhodopseudomonas sphaeroides (10). In addition, in our own laboratory we have found an isoschizomer of PvuI to be present in the organism Xanthomonas nigromaculans, ATCC 23390, (XnlI) (R.E. Gelinas, P. Bullock, and R.J. Roberts, unpublished observations). However, the latter organism produces rather small amounts of XnlI and the enzyme from that source is difficult to purify. In the case of both XorII and RshI, the site of cleavage within the recognition sequence is identical with that found for PvuI. No other Type II restriction enzyme has been described with the specificity of PvuII and so this constitutes a valuable new addition to the collection of specific endonucleases.

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REFERENCES