Characterization of the Type III restriction endonuclease PstII from *Providencia stuartii*

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ABSTRACT

A new Type III restriction endonuclease designated PstII has been purified from *Providencia stuartii*. PstII recognizes the hexanucleotide sequence 5’-CTGATG(N)25-26/27-28-3’. Endonuclease activity requires a substrate with two copies of the recognition site in head-to-head repeat and is dependent on a low level of ATP hydrolysis (~40 ATP/site/min). Cleavage occurs at just one of the two sites and results in a staggered cut 25–26 nt downstream of the top strand sequence to generate a two base 5’-protruding end. Methylation of the site occurs on one strand only at the first adenine of 5’-CATCAG-3’. Therefore, PstII has characteristic Type III restriction enzyme activity as exemplified by EcoPI or EcoP15I. Moreover, sequence asymmetry of the PstII recognition site in the T7 genome acts as an historical imprint of Type III restriction activity *in vivo*. In contrast to other Type I and III enzymes, PstII has a more relaxed nucleotide specificity and can cut DNA with GTP and CTP (but not UTP). We also demonstrate that PstII and EcoP15I cannot interact and cleave a DNA substrate suggesting that Type III enzymes must make specific protein–protein contacts to activate endonuclease activity.

INTRODUCTION

Of the three major Restriction-Modification (RM) systems, the Type III enzymes are the least well characterized. A combination of genetics and bioinformatics has revealed, to-date (1), >140 confirmed and putative Type III RM systems not including orphan open reading frames (ORFs). These represent ~6% of all RM systems, although, the true percentage may be higher due to the over-representation of commercially exploitable Type II enzymes. The vast majority of biochemical studies on Type III RM enzymes have been undertaken using two closely related protein complexes, EcoPI (Please note that by common agreement the nomenclature EcoPI replaces the alternatives EcoPII and EcoPI) and EcoP15I (2–4). Other enzymes that have been analysed to a lesser degree include HinIII, HinI (5–7), StyLI (8,9), LlaFI (10), BceS1 (11) and PhaB1 (1,12). A general model for DNA modification and cleavage by all Type III enzymes has been proposed (2–4), based partly on a general model that has been established for the related Type I RM enzymes (3). In contrast to these unified Type I and III schemes, the current view of the Type II RM enzymes is that the DNA cleavage is accomplished by a multiplicity of different mechanisms, many of which are very different from the activities of archetypal enzymes such as EcoRI and EcoRV (13). Indeed it now appears that the majority of Type II enzymes must bind to multiple recognition sequences in order to cut DNA. This new orthodoxy has only been revealed by the analysis of an expanded range of enzyme systems [e.g. even when recognizing the same DNA sequence, many different Type II mechanisms have evolved, (14)]. Therefore, one could question whether it is appropriate to invoke a single mechanistic model for all Type III enzymes given the paucity of in-depth comparative studies. Do alternative Type III activities exist? Here, and in the accompanying paper (15), we describe the detailed characterization of a new Type III RM system, the PstII restriction enzyme from *Providencia stuartii*. From our results it appears that the PstII RM system conforms to the general Type III model and historical evidence of its prototypical Type III restriction activity can be inferred from the genome sequences of some bacteriophage.

MATERIALS AND METHODS

DNA substrates

Unless stated otherwise, all DNA manipulations were carried out using standard procedures (16). All oligonucleotides were...
supplied by MWG Biotech (Germany). Two-site DNA substrates were created by direct cloning of the following duplex oligonucleotide into the appropriate sites of pNAG4 (17) or pAT153 (18): 5'-AGCTGACGACCATCAGGGACACGTCGAG-3' was annealed to 5'-AGCTCTCGAGCTGTCGCTGATGGTCGTC-3' (PstII sequence underlined), and the duplex cloned into the HindIII site of pNAG4 to give pLJP11a or pLJP11b depending upon the orientation of the PstII site (Figure 1A); 5'-AGCTGATGAACTGCCTTGCTACAGTGTTGGCTAGAAC-3' was annealed to 5'-AGCTGTTCTAGCCAGCTGTAGCAAGGCAGTTATC-3' (PstII sequence underlined), and the duplex cloned into the HindIII site of pAT153 to give pLJP12c (sites chosen to be in indirect repeat); and, 5'-AGCTGATGCTTTCTAAGGGTAATTTTAAAATTATCT-3' was annealed to 5'-AGCTAGATATTTTAAAATATCCTTAGAAAGCATC-3' (PstII sequence underlined), and the duplex cloned into the HindIII site of pAT153 to give pLJP12d (sites chosen to be in indirect repeat). *Escherichia coli* TOP10 cells (Invitrogen) were transformed with the plasmids and the covalently closed circular (CCC) form of the DNA purified by density gradient centrifugation in CsCl-ethidium bromide (19). Where labelled DNA was required, the transformants were grown in M9 minimal medium supplemented with 37 MBq/l [3H-methyl] thymidine. Linear DNA substrates were generated from the plasmid by digestion with the appropriate Type II restriction enzyme and the DNA purified by phenol/chloroform and chloroform extraction followed by ethanol precipitation. DNA concentrations were determined from UV absorbance at 260 nm, assuming that an OD₁₀₀₀ corresponds to 50 µg/ml DNA and a molecular weight of 6.6 x 10⁵ Da/kb.
Cloning of PstII RM system

E. coli cells expressing the cloned PstII RM system were selected from a genomic library of P. stuartii DNA by their ability to restrict bacteriophage as follows. Total cellular DNA of P. stuartii 164 was purified, digested with HindIII, ligated into similarly-cleaved pBR322 (20), and competent E. coli RR1 cells (16) transformed with the clones. Transformed colonies were grown overnight at 37°C on plates containing ampicillin (50 μg/ml), and collectively harvested to make the cell library (21). Aliquots of this library, containing ~1 x 10^9 cells, were spread onto ampicillin-plates together with ~1 x 10^9 particles of T4GT7-cyt, and incubated overnight at 37°C. T4GT7-cyt is a derivative of T4 bacteriophage that contains cytosine in its DNA in place of the glucosylated hydroxymethylcytosine present in wild-type T4. The absence of the glucosylated hydroxymethyl modification renders the hydroxymethylcytosine present in wild-type T4.

Colonies expressing the cloned PstII RM system were selected from a genomic library of P. stuartii containing a fragment of a normal morphology and smooth margins carried plasmids areed plasmids containing a fragment of ~4.0 kb, colonies with a normal morphology and smooth margins carried plasmids containing a fragment of ~6.1 kb. The former were shown to encode the PstI RM system (24), and the latter to encode the PstII RM system (see below).

Clone characterization

Colonies of both kinds were assayed for endogenous restriction endonuclease activity. Cell-extracts were prepared, serially diluted, incubated for 1 h at 37°C in 8 ml molten top agar and poured onto Tryptone agar plates containing 10^7 particles/ml in water. Sequencing reactions comprised 5 μl of template DNA (0.5 μg), 1 μl of primer (3.2 pmol) and 4 μl AmpliTaq DNA polymerase FS premix (AB PRISM dye terminator cycle sequencing ready reaction kit; Applied Biosystems). The reactions were prepared in 96-well plate format using a Biomek 2000 robot (Beckman) and thermocycled 25 times according to the standard AB protocol. The products were purified on Centrisep spin-columns, vacuum-dried, resuspended in 2 μl Accutrac dye, heated to 90°C for 2 min and analysed in an AB 377 sequencer (25). Raw sequence runs were edited and assembled using ‘Autoassembler’, formatted with ‘Gene Jockey’, and analyzed using the web-based BLAST programs at NCBI (http://www.ncbi.nlm.nih.gov) (26) and REBASE (1).

Purification of PstII

PstII was purified from E. coli TOP10 cells transformed with one of the recombinant clones from the sequencing study (p4409). Typically, cells were grown in 1 l of LB medium containing ampicillin (50 μg/ml) at 37°C and 245 r.p.m. until an OD_{600} of 2.0. The harvested cells were resuspended in 20 ml of Buffer A [20 mM Tris–HCl (pH 8.0), 1 mM EDTA, 10% (v/v) glycerol, 100 mM NaCl, 0.5 mM DTT] and disrupted by sonication on ice. The cell extract was clarified by low speed centrifugation at 23,000 g for 10 min followed by 100,000 g for 2 h. The supernatant was dialysed against Buffer B [50 mM Tris–HCl (pH 8.0), 1 mM EDTA, 50 mM NaCl, 1 mM DTT] and the filtered dialysate loaded onto 20 ml Hi-prep heparin Sepharose FF columns (Amersham Biosciences) connected in series and equilibrated in Buffer B. Bound proteins were eluted with a linear gradient of NaCl in Buffer B (0.10–0.75 M, 420 ml). Fractions were analysed for DNA cleavage activity on pLJP11b and by SDS–PAGE. PstII was eluted in a peak at ~0.41 M NaCl. Significant loss of Res subunit was noted (Res eluted in a far broader peak than Mod). Those fractions containing approximately equal proportions of both Res and Mod were pooled and further purified on an 8 ml Mono-Q column (Amersham Biosciences) equilibrated with Buffer B. PstII was eluted with a linear gradient of NaCl in Buffer B (0.10–1.00 M, 200 ml). Fractions were analysed for DNA cleavage activity on pLJP11b and by SDS–PAGE. PstII eluted in a peak at ~0.43 M NaCl with little separation of Res and Mod subunits. The fractions containing pure Res and Mod (>95% homogeneity) were pooled and concentrated by spin filtration in Amicon Ultra-15 columns (30 000 MWCO, Millipore). The final retentate was supplemented to 50% (v/v) glycerol and stored at ~20°C with no loss of activity upon storage noted. Protein concentration was determined from the UV absorption at 280 nm using an extinction coefficient derived from the aromatic amino acid composition of the predicted amino acid sequences. All PstII concentrations are quoted in terms of a mixture of Res and Mod subunits in the ratio 1:3 as estimated from densitometry of Coomassie-stained SDS gels and assuming a linear relationship between protein size and dye intensity.

DNA sequencing and analysis

The nucleotide sequence of the 6.1 kb fragment was determined by ‘primer-walking’ using synthetic 20–24 nt primers (New England Biolabs Organic Synthesis Division, Beverly MA). Sequencing started on each side of the HindIII site of pBR322 and proceeded into the recombinant fragment in both directions, using the nucleotide sequence obtained in each cycle to design the primer for the next. Template DNA was purified using Qiagen midi-columns and resuspended to 100 μg/ml in water. Sequencing reactions comprised 5 μl of template DNA (0.5 μg), 1 μl of primer (3.2 pmol) and 4 μl AmpliTaq DNA polymerase FS premix (AB PRISM dye terminator cycle sequencing ready reaction kit; Applied Biosystems). The reactions were prepared in 96-well plate format using a Biomek 2000 robot (Beckman) and thermocycled 25 times according to the standard AB protocol. The products were purified on Centrisep spin-columns, vacuum-dried, resuspended in 2 μl Accutrac dye, heated to 90°C for 2 min and analysed in an AB 377 sequencer (25). Raw sequence runs were edited and assembled using ‘Autoassembler’, formatted with ‘Gene Jockey’, and analyzed using the web-based BLAST programs at NCBI (http://www.ncbi.nlm.nih.gov) (26) and REBASE (1).

DNA cleavage assays with PstII and/or EcoP15I

Cleavage reactions contained 5 nM plasmid DNA or 500 ng phage DNA, and 129 nM PstII mixture (or 1/10 vol lysate/column fraction), and/or 25 nM EcoP15I in NEB 4 [20 mM
Tris-acetate (pH 7.9), 10 mM Mg acetate, 50 mM K acetate, 1 mM DTT] supplemented with AdoMet and NTPs as indicated in the text. When PstII was added the buffer was further supplemented with 0.02% (v/v) Triton-X100. Reactions were started by addition of Type III enzyme(s) to reduce the effect of co-methylation upon pre-incubation. Reaction aliquots were quenched after the required incubation times by addition of 0.5 vol of STEB [0.1 M Tris–HCl (pH 7.5), 0.2 M EDTA, 40% (w/v) sucrose, 0.4 mg/ml bromophenol blue]. The CCC DNA substrate, open circle/nicked intermediate (OC) and full-length linear product (FLL) were separated by agarose gel electrophoresis and the percentage of DNA in each band evaluated by scintillation counting (19). Where shown, digital images of the agarose gels were captured on a Kodak Image Station 440CF without editing and using a linear intensity scale.

DNA methylation with PstII

To generate substrates for methylation studies, oligonucleotides were obtained from MWG Biotech. Two of these were non-methylated whilst three were methylated at one of the three putative target adenine residues in the PstII recognition sequence. 5'-AGATGACGACCATCGAGGACAGCTTCAGGATCGCTCGCGGCTCTTACC-3' was annealed to 5'-GGTAAGAGCCGCGAGCGATCCTTGAAGCTGTCCCTGATGGTCGTCATCT-3' (PstII sites underlined, methylated adenine resides bold type). Methylation reactions contained 50 μM duplex oligonucleotide and 129 nM PstII mixture in NEB 4 supplemented with 0.465 mM [3H-methyl] AdoMet and 0.02% (v/v) Triton-X100. Reactions were incubated for 3 h at 37°C. The DNA was phenol chloroform extracted and ethanol precipitated and the extent of [3H-methyl] transfer quantified by scintillation counting.

Identification of the PstII recognition sequence

An arbitrary plasmid library was obtained as target DNA substrates (unpublished data) and digested with PstII for 1 h at 37°C with 4 mM ATP. For those plasmids which were cleaved, the locations of the break sites were mapped to within ±100 bp following secondary cleavage with suitably located Type II restriction enzymes. Computer analysis using ‘SequenceMap’ (Allan Brewer, University of Bristol) was then undertaken to compare all 5 or 6 bp DNA sequences common to the cleavage regions. Of the candidates identified we rejected all the directly-repeated sites and all indirectly-repeated sites that appeared on substrates which could not be cut. This narrowed down the search to three 6 bp sequences. A second plasmid library was then methylated by PstII in the presence of [3H-methyl] AdoMet and in the absence of ATP. The circular DNA were cleaved with suitably located Type II restriction enzymes to generate a characteristic ladder of linear DNA and these scintillation fragments counted following separation by gel electrophoresis. The various patterns of labelled fragments obtained could only be explained by methylation of the CTGATG sequence. Final confirmation came from analysis of specific plasmid and oligonucleotide substrates (Figure 2).

Figure 2. DNA sequences and substrates for the PstII restriction enzyme. (A) Recognition sequences for PstII and EcoP15I. The adenine residue methylated is underlined. The non-specific cleavage loci are indicated by arrows. The arbitrary direction of the sites is indicated by the arrowhead (black/white) and is set by the location of the cleavage site relative to the recognition site. (B) DNA substrates used in PstII assays with total sizes in brackets (bp). Distances between sites represent nucleotide spacings not including the base pairs in the recognition sequence. Sites with different flanking sequences are indicated by ‘1’ or ‘2’ (see Figure 3). (C) Cleavage of DNA substrates from (B) with PstII as indicated (see main text for more details). The resulting fragments were separated by agarose gel electrophoresis. The locations of the intact plasmid and cleavage products are indicated [note that the plasmids differ in size, (B)]. For LinHtH, the cleavage of either site 1 or 2 produces a characteristic pair of DNA fragments.
Identification of the PstII cleavage loci

Sequencing was performed using Sequenase 2.0 (USB). All primers were supplied by Sigma Genosys: 5'-GCCTACAATCCATGCAAAC-3' was used for site 1 and 5'-TGCCACCTGACGTCTAGAA-3' was used for sites 2, 3 and 4. Plasmid DNA was alkali denatured and then precipitated. The primer extension reaction contained 1.2 pmol denatured DNA and 2 pmol primer in NEB 4. The primer was annealed by heating to 90°C and cooling to 20°C for 15 min. Extension was started by addition of 2 nmol dNTPs, 1.68 nmol [α-32P]-ATP and 10 U Klenow polymerase (Fermentas). Reactions were incubated at 37°C for 15 min then heat inactivated at 75°C for 20 min. PstII mixture (15.5 pmoles) and 4 mM ATP were added, and the reactions incubated for 30 min at 37°C, and then heat inactivated at 67°C. The reactions were then split into two and one-half treated with 200 U dNTPs and 1 U Klenow polymerase at 20°C for 10 min. Reactions were stopped with the Sequenase loading buffer. Sequencing reactions were run alongside the primer extension reaction on a denaturing PAGE [6% (v/v) polyacrylamide]. Images were captured using a Molecular Dynamics Typhoon 9200 PhosphorImager.

Coupled NTPase assay

Measurement of NTPase activity was carried out using a coupled assay according to Kijanitsa et al. (27). Reactions (200 μl) contained 129 mM PstII mixture, 1 mM plasmid DNA (pAT153 or pSKFoK1), 4.40 U lactate dehydrogenase (Sigma–Aldrich), 2.91 U pyruvate kinase (Sigma–Aldrich), DNA (pAT153 or pSKFoK1), 4.40 U lactate dehydrogenase, 0.80 U phosphoglycerate kinase (Sigma–Aldrich), 2.91 U pyruvate kinase, and 10 U Klenow polymerase (Fermentas). Reactions were incubated for 30 min at 37°C, and then heat inactivated at 67°C. The reactions were then split into two and one-half treated with 200 U dNTPs and 1 U Klenow polymerase at 20°C for 10 min. Reactions were stopped with the Sequenase loading buffer. Sequencing reactions were run alongside the primer extension reaction on a denaturing PAGE [6% (v/v) polyacrylamide]. Images were captured using a Molecular Dynamics Typhoon 9200 PhosphorImager.

RESULTS

Isolation of the PstII RM system

The PstII RM system was discovered when a plasmid library of P. stuartii 164 DNA was screened in E. coli for cloning that restricted the growth of bacteriophage. Restricting cells can proliferate in the presence of certain phage, whereas non-restricting cells succumb to infection and die (24). E. coli cells harbouring the library were exposed to a restriction-sensitive derivative of the virulent, T4 bacteriophage (Materials and Methods). Two kinds of restricting clones survived. One carried a 4.0 kb fragment that encoded the Type II RM system PstI (24) and the other carried a 6.1 kb fragment that encoded a different RM system, the existence of which was hitherto unknown. We designated this second system PstII (1). The two restricting systems were shown to differ by biochemical and phenotypic tests. Cell extracts of each were prepared and assayed for restriction endonuclease activity. Extracts of PstI-clones contained abundant endonuclease activity that produced DNA-digestion patterns characteristic of that enzyme.

Table 1. Approximate efficiencies of plating (eop) of phage λ and Φ80 derivatives on E. coli harbouring cloned RM systems

| Phage | Modification | Efficiency of plating |
|-------|--------------|-----------------------|
|       |              | Host restriction system |
|       |              | Control | PstI | PstII | EcoRI |
| λ Cl- | None         | 1           | 10⁻⁵  | 10⁻⁵  | 10⁻²  |
| λ vir | None         | 1           | 10⁻⁵  | 10⁻⁵  | 10⁻⁴  |
| λ vir | PstII        | 1           | 1     | 10⁻⁶  |
| Φ80 CI-| None        | 1           | 10⁻⁵  | 10⁻⁶  |
| Φ80 CI-| PstII       | 1           | 1     | 10⁻⁶  |

Phage grow with an eop of one on cells harbouring no RM system (control). Unmodified phage are severely restricted (grow with much-reduced eop) on cells carrying either the PstI or PstII systems from P. stuartii, or the native EcoRI system. Rare phage that escape restriction acquire DNA-modification that protects them from subsequent restriction by that host. PstI-modified phage continue to be restricted by PstI, however, and PstII-modified phage continue to be restricted by PstII, indicating that the protective modification is systemspecific, and that the specificities of the two systems are therefore different.

Extracts of PstII-clones, in contrast, produced barely detectable banding patterns that defied immediate identification. However, cells of both types restricted lambdoid phages efficiently (Table 1), implying that both RM systems were expressed and active in vivo.

Rare plaques from phages that had escaped restriction were isolated from cells of both types, and propagated on the same cells to prepare phage-stocks bearing system-specific modifications. PstI-modified phage, no longer restricted by PstI-containing cells, continued to be restricted by PstII. Conversely, PstII-modified phage, no longer restricted by PstII, continued to be restricted by PstI (Table 1). These results implied that both systems were complete and could modify as well as restrict, and that the sequence-specificities of the PstI and PstII systems were distinct.

Sequence and analysis of the PstII RM system

The nucleotide sequence of the 6.1 kb fragment was determined (GenBank accession no. DQ143901). The PstII system occupies the central 4.5 kb of this sequence and comprises two adjacent, similarly oriented genes, pstIIIM (1685 bp) and pstIIIR (2858 bp) coding for the Mod (561 amino acid) and Res (952 amino acid) subunits of what sequence comparisons suggested was an RM system of the Type III class (1). In addition to the PstII operon this fragment also contains one additional ORF encoded a putative 182 amino acid polypeptide (data not shown); this was not readily identified and was not investigated further. Type III RM operons consist of two genes, mod and res, which lie closely adjacent to each other and are transcribed in the same direction with mod preceding res. The genes encode two polypeptide subunits, Mod and Res, respectively (1–4). Mod.PstII has amino acid motifs [Figure 1, (28)] and predicted secondary structural features (data not shown) characteristic of an α-group methyltransferase (29). These include: Motif I, which is likely to be involved in binding of the methyl donor S-adenosyl methionine (AdoMet); Motif IV, which is likely to be involved in the catalysis of DNA methylation at the exocyclic N6 of adenine; and a target recognition domain (TRD), which is likely to recognize a
specific DNA sequence. Homodimeric forms of Mod.EcoP15I and Mod.EcoP15I (Mod.) have been shown to act independently as methyltransferases, both recognizing and modifying their target sequences independent of Res (30,31). The Mod.PstII sequence shares amino acid features with the methyltransferase regions of Mod.EcoP15I and Mod.EcoPI (~30% identity, ~46% similarity, data not shown). The pstIIIR overlaps pstIM by 4 nt. A similar arrangement is seen in other Type III systems (1), although mod and res genes may actually be expressed from separate promoters rather than as a polycistronic transcript (32). Res.PstII has amino acid motifs [Figure 1, (28)] and predicted secondary structural features (33) characteristic of both an endonuclease (Region X) and a superfamily 2 DNA helicase. The Type III helicase motifs can be classified on the basis of sequence alignments and predicted secondary structures in one of two groups, Type IIIA and Type IIIB (33). Whilst Res.EcoP15 and Res.EcoPI are Type IIIA, Res.PstII falls into the Type IIIB group (alignments are shown for helicase Motif I in Figure 1B).

The recognition site of PstII
To ascertain the recognition sequence of PstII we chose to use an in vitro approach (Materials and Methods). Our first step was to purify the complete RM system from E.coli TOP10 cells transformed with one of the PstII clones (Materials and Methods, Figure 1C). The final purified samples contained a Res:Mod ratio of ~1:3. In the accompanying paper (15), we show that maximum PstII endonuclease activity is likely to be obtained with a Res2Mod2 assembly as shown for EcoP1I/EcoP1SI (34). However, in contrast to the E.coli complexes, assembly of the PstII heterotetramer is dynamic and the reaction conditions used in this paper (Materials and Methods) were chosen to favour saturation of the DNA sites with the Res2Mod2 form rather than sub-assemblies that have reduced endonuclease activity and enhanced methyltransferase activity (15). All concentrations of the PstII mixture were quoted in terms of a Res:Mod ratio of ~1:3. We observed that unregulated expression of the genes from the native promoters on a high copy number plasmid resulted in low transformation efficiencies and that some transformants contained inactive protein (data not shown). It would appear that horizontal transfer of PstII can result in cell death (most likely due to DNA breakdown). This is similar to observations with StyLTI (8) but is in contrast to the more freely-transferable EcoP1I and EcoP1SI genes [(35) and references therein]. In these latter cases restriction activity is delayed until methylation is complete as Mod regulates the expression of Res with additional control at the translational level.

Mapping using both DNA cleavage and methylation activities identified the recognition sequence of PstII as CTGATG (Materials and Methods, Figure 2A). This analysis relied partly on the assumption that PstII endonuclease activity requires two indirectly-repeated copies of its recognition site as shown for other Type III enzymes such as EcoP1I and EcoP1SI (36,37). For instance, for an EcoP1SI substrate to be cleaved it must carry a 5'-CAGCAG-3' sequence (Figure 2A) followed by a 5'-CTGCTG-3' sequence on the same strand—any other arrangement is incompatible with cleavage expect under certain special conditions. To confirm that this assumption is true for PstII we measured cleavage activity on DNA substrates with different numbers of CTGATG sites (Figure 2B); either zero sites [pSKFokI, (38)], one site [pAT153, (18)], two directly-repeated sites (pLJP11a) or two indirectly-repeated sites (pLJP11b). The only substrate that could be cleaved in the presence of 4 mM ATP was pLJP11b (Figure 2C). No cleavage was seen with 4 mM ATPyS (data not shown). The presence or otherwise of 100 μM AdoMet did not affect the outcome of the reactions under these conditions. The final product of the reaction was the FLL DNA cut at one or other site (the cleavage loci were mapped precisely as described below, Figure 3). No further
cleavage was observed at the remaining intact site. On a circular DNA the indirectly-repeated sites can be classified as being in both ‘head-to-head’ and ‘tail-to-tail’ orientations [the definition of the ‘direction’ of the site (Figure 2A) is due in part to the location of the cleavage loci, see below]. To distinguish between these arrangements we measured cleavage activity on two linear versions of pLJP11b in which the sites are in either head-to-head (linHtH) or tail-to-tail (linTtT) arrangement (Figure 2B). Of these, only linHtH was cleaved (Figure 2C) at either site 1 (to produce 1822 and 2445 bp fragments) or site 2 (to produce 1390 and 2877 bp fragments); the 1055 bp fragment that would be released if both sites were cut was never observed. A preference for site 1 over site 2 was never observed. A preference for site 1 over site 2 was observed (in the ratio 68:32, data not shown) which is likely to be due to differences in the flanking non-specific DNA sequences (Figure 3). Similar preferences in both cleavage and methylation have been observed for other Type III enzymes using sites with dissimilar flanking sequences (6,39). When flanking sequences are identical, cleavage is divided equally between the two sites [e.g. for EcoPI, (36)].

Another assumption of the mapping experiments was that DNA cleavage occurred at non-specific sites immediately adjacent to the recognition site (Materials and Methods). To characterize the cleavage sites more in detail we mapped the PstII strand breaks using the sequencing approach of Janulaitis et al. (40). In brief, the site 2 plasmid substrates pLJP11b, pLJP12c and pLJP12d were subjected to primer extension in the presence of [α-32P]ATP to generate dsDNA with one labelled strand. Each primer was extended over the DNA arc containing the sites in head-to-head orientation so that the labelled DNA was a substrate for PstII (i.e. equivalent to linHtH in Figure 2C). This DNA was cut with PstII and the sample split into two aliquots which were analysed by denaturing PAGE; one aliquot was loaded directly onto the gel whilst the other was first treated with Klenow polymerase (Materials and Methods). Where the ‘top’ (5’-3’) strand is labelled, the first sample gives the location of the cleavage site on the top strand. The length of the second sample relative to the first then reports on the cleavage of the unlabelled bottom (3’-5’) strand: if cleavage generates a 5’-overhang, then the top strand will be extended; if cleavage generates a 3’-overhang, then the top strand will be digested back; if cleavage generates a blunt end, then no change in length will be observed. We analysed four PstII sequences that differed in their flanking sequences (Figure 3A): sites 1 and 2 from pLJP11b, site 3 from pLJP12c and site 4 from pLJP12d (pLJP12c and pLJP12d also carry site 1 which was not examined). Cleavage of the top strand mapped to non-specific loci 25–26 bp 3’-to the recognition site. In every case treatment with Klenow polymerase generated a fragment extended by 2 nt indicating a 5’-overhang 2 nt in length. These cleavage loci and their relative locations are similar to those observed for EcoP15I ([41], Figure 1A). The 1 nt difference in spacing of the cleavage site at site 1 compared to the other sequences is likely to be due to DNA structural features local to the scissile phosphodiester bonds (39) and/or the exact number of base pairs per helical turn in the intervening DNA. As well as the Type III enzymes, some Type IIS and IIB enzymes that also cut non-specific DNA adjacent to their sites have been shown to cleave imprecisely (1,40).

To pinpoint the recognition site residue(s) methylated, we measured the modification of a series of synthetic oligonucleotide substrates using [3H-methyl] AdoMet (Materials and Methods, Figure 4). Janulaitis et al. (40) have used a similar procedure with Type II enzymes, e.g. AloI. Each dsDNA substrate was treated with PstII for 3 h in the presence of [3H-methyl] AdoMet and in the absence of NTPs. Under these conditions methylation will occur without any DNA cleavage (data not shown). Excess AdoMet was removed and the extent of labelling ascertained by scintillation counting. We first analysed two unmodified DNA substrates; a non-specific DNA and a specific DNA carrying the PstII recognition site (Figure 4A). The specific DNA was modified to a significantly greater degree than the unmodified DNA (>1.6 × 10^3 DPM compared to <2.0 × 10^3 DPM with background at 0.113 × 10^3, Figure 4B). Secondly we analysed three pre-modified oligonucleotides in which an N6-methyl deoxyadenosine phosphoramidite was incorporated during synthesis at one of the three target adenine residues in the PstII recognition sequence (Figure 4A). When the first adenine on the 5’-CATCAG-3’ strand was pre-methylated (position 3), PstII transferred very little radioactivity to the DNA (<7.0 × 10^2 DPM, Figure 4B). In contrast, pre-methylation of either of the other two adenine residues in the PstII sequence (positions 1 or 2) resulted in labelling at a level similar to that observed on the unmodified specific DNA (>1.5 × 10^3 DPM). Therefore, we conclude that PstII only hemi-methylates its recognition sequence at one
adenine residue on one strand (Figure 2A), in a similar manner to other Type III enzymes (42,43).

The PstII experiments illustrates a number of key points: (i) DNA cleavage requires a pair of recognition sites in cis on the same DNA molecule; (ii) the two sites must be arranged ‘head-to-head’ such that the sites appear on one strand as 5’-CTGATG-3’ followed by 5’-CATCAG-3’; (iii) hydrolysable ATP, but not AdoMet, is absolutely required for cleavage; (iv) only one of the two sites is ever cleaved; (v) DNA cleavage occurs 25–26 bp downstream of the CTGATG strand and generates a 2 bp 5’-overhang; (vi) methylation of the site occurs on only one strand at a specific adenine residue. These are precisely the results predicted by the general Type III model. We also demonstrate in the accompanying paper (18) that DNA cleavage only results when a stoichiometric or greater amount of protein is added suggesting that, as with other Type III enzymes, PstII cannot turnover once it has cut the DNA. We tested a number of other buffers (both K+ and Na+-ion based) but did not observe any differences in the cleavage characteristics (data not shown). We did not observe any secondary (2°) cleavage events. 2° events occur with EcoPI and EcoP15I in the presence of excess enzyme under relaxed buffer conditions, in particular the absence of the specificity factor AdoMet (36). It may be that because of the weak association of the Res and Mod subunits in PstII (15), we cannot add sufficient tetrameric enzyme to our in vitro experiments to activate a 2° event.

DNA cleavage activity

The rate of DNA cleavage of the supercoiled 2-site substrate pLJP11b was analysed (Figure 5). The reactions were initiated by adding an excess of PstII to DNA sites to favour formation of Res2Mod2 complexes (Materials and Methods). Although this may result in a slow second order binding step preceding the cleavage kinetics, we have adopted this approach in general with Type III enzymes (36,44) to avoid co-methylation inhibiting the reaction before we can start to measure cleavage. The rate of digestion of pLJP11b was rapid compared to other Type III (and Type I) restriction enzymes (36,45) with the majority of the substrate cleaved in ~10 s at 37°C. AdoMet did not affect this rate (the rate of appearance of FLL DNA is compared in Figure 5). Even at temperatures down to 4°C, PstII had relatively high endonuclease activity (data not shown). At all temperatures the intermediates nicked in one or other strand did not accumulate significantly during the reaction and the CCC substrate was converted directly to the FLL product. Similar profiles have been observed with EcoP15I and EcoPI (36,39,46). Given that Type III enzymes only work under ‘single turnover’ conditions ([protein] ≫ [DNA sites]), these profiles can be explained by one of two simple models, either: (i) that the rate for cleaving the first strand is slower than that for cleaving the second; or, (ii) that there is a rate-limiting step prior to cleavage of the first strand. We did not observe any differences in the profiles when reactions were carried out using Na+-based buffers (data not shown).

NTPase activity

Given the Type IIB helicase motifs in the Res.PstII subunit and the ATP-dependent DNA cleavage shown in Figures 2 and 5, it was expected that PstII would have an ATPase activity equivalent to those measured previously for other Type III enzymes (47,48). We first investigated the ability of a range of nucleotide triphosphates to activate DNA cleavage by PstII and EcoP15I (Figure 6A). A total of 5 nM pLJP11b (for PstII) or pMDS34a (for EcoP15I, 44) were incubated for 1 h at 37°C with excess enzyme and 4 mM nucleotide, as indicated. In addition to ATP, DNA cleavage by PstII was also activated by dATP, ddATP, GTP, ddGTP, CTP and all forms of UTP/TTP failed to activate cleavage. In comparison, EcoP15I was only activated by adenosine triphosphates (Figure 6A) as observed previously (49). Some 2° cleavage of the DNA was observed with EcoP15I (these are relaxed buffer conditions). As above, no secondary cleavage was observed with PstII. Although hydrolysis of a full range of NTPs, dNTPs and ddNTPs has been observed for other DexH- and DEAD-box helicases (50), to the best of our knowledge this is the first report of a Type III (or Type I) restriction enzyme that can utilise nucleotides other than those based on adenosine. Given the similarities in the ATP binding affinities of PstII and EcoP15I (see below), we do not believe that ATP contamination can explain why PstII is active with GTP or CTP when EcoP15I is not. Hydrolysis of the ddNTPs is difficult to measure using our coupled assay (Materials and Methods) because the coupling enzymes become rate-limited. Since ATP;S does not facilitate cleavage, we do not believe that binding alone by dNTPs or ddNTPs is sufficient but, instead, that hydrolysis must occur.

The dependence of the cleavage reaction on the relative concentrations of the NTPs was examined by measuring the quantity of FLL DNA liberated following cleavage for 1 h at 37°C (Figure 6B). DNA cleavage was complete at ATP concentrations above ~3 μM whilst two-orders of magnitude more CTP or GTP was required, with a slight preference
for CTP over GTP. Cleavage was not observed at any concentration of UTP (data not shown). Similar sigmoidal cleavage profiles have been observed for EcoP15I with a $K_{1/2}$ of $\approx 7\mu M$ (49,51). The ranking order of ATP $>$ CTP $>$ GTP was also observed by measuring the rates of DNA cleavage at 4 mM rNTP (the appearance of FLL DNA is compared in Figure 6C), although the differences between ATP, CTP and GTP were not as marked as in Figure 6B. Where cleavage...
occurred, the CCC substrate was always converted directly to FLL product with no build-up of the OC intermediate (full profiles not shown).

The rate of rNTP hydrolysis was measured at a range of concentrations using a continuous coupled assay (Materials and Methods). No activity was observed in the absence of polynucleotide (data not shown). As DNA substrates we utilized either a zero-site plasmid pSKFok1, (38) or a one-site plasmid pAT153, (18). Using 2-site DNA did not produce more than a linear increase in Type III NTPase activity (data not shown)—there is no apparent cooperativity when multiple sites are present. The rate on the specific DNA (NTP hydrolysed per DNA site per min) was corrected for the substantial background rate on the non-specific DNA (Figure 6D). As elsewhere in this paper we utilized a high concentration of the PstII mixture (129 nM) to favour formation of the tetramer (15). This significant excess of enzyme over sites is likely to be the cause of the high background rate. Combined with the low levels of ATPase activity observed, this resulted in correspondingly high levels of experimental deviation such that some apparent negative rates were recorded (this is where the background rate was higher that the specific rate). It should also be noted that due to limitations of the experimental set-up, rates could only be collected over tens of minutes whereas cleavage would have finished in tens of seconds (Figure 6C). Therefore, it is not clear how the observed rates relate to the true NTP consumption preceding a cleavage event. Nevertheless, the apparent NTPase rates in Figure 6D show a pattern similar to those in Figure 6B and C in that ATP hydrolysis was favoured over CTP or GTP hydrolysis. The relatively slow NTPase rates observed (Figure 6D) are similar in magnitude to the ATPase rates measured previously with EcoPI and EcoP15I (47,48). Very little UTP hydrolysis could be measured. However, by titrating increasing concentrations of UTP (0–8 mM) against a fixed concentration of ATP (4 mM), an inhibition of the cleavage reaction was observed (Figure 6E). It suggests that UTP can compete with ATP for the nucleotide binding site but that UTP cannot be coupled efficiently to hydrolysis or DNA cleavage. The NTPase kinetics observed did not clearly match a Michaelis–Menten relationship (data not shown). The true relationship is being investigated in more detailed studies (A. Sears and M. D. Szczelkun, unpublished data). Non-Michaelis–Menten kinetics have also been observed with EcoPI (48).

**Long-distance interactions between enzymes**

The data in this paper shows that PstII must communicate between a pair of distant sites in head-to-head orientation in order to cleave a DNA substrate. It has been suggested for the Type I RM enzymes that DNA cleavage can be initiated at any site where a translocating enzyme stalls (52,53). A specific protein–protein interaction may not be required as two unrelated Type I enzymes can mutually activate cleavage of a linear DNA which is not cut by either enzyme alone (52). Similarly, an interaction between EcoPI and EcoP15I has been shown to result in mutual activation of cleavage (34,54). However, the EcoPI and EcoP15I proteins are so similar in sequence as to be considered, fundamentally, as the same enzyme. Therefore, it is not clear if the communication between Type III tetramers require a specific protein–protein interaction or if a simple stall in translocation is sufficient.

To address the communication question we tested if the less closely related PstII and EcoP15I enzymes could interact to cleave T7 phage DNA (Figure 7). It has been noted previously that T7 phage DNA is refractory for cleavage by EcoP15I as all 36 recognition sites are in the same directly-repeated orientation (37,47,55,56). Strand bias is also true of the 34 PstII sites [Table 1, (55)] and, accordingly, T7 DNA is refractory to cleavage by PstII (Figure 7). The PstII and EcoP15I recognition sites share a common sequence motif, CANCAG. Significant strand bias is also observed in the other two versions of this motif (Table 1). The possible significance of this observation is discussed more in detail below. Using the definition of Type III site directionality as shown in Figure 2A, the PstII and EcoP15I sites in T7 can be said to be in a head-to-head orientation (Figure 7A). We utilized this fortuitous arrangement to test if a mixture of the two could interact and mutually activate cleavage. Lambda phage DNA in which strand bias of PstII and EcoP15I sites is not observed was utilized as a control (Figure 7A).

On T7 DNA, neither PstII alone nor EcoP15I alone generated any double-strand breaks, even after extensive incubation (Figure 7B, lanes 8–10). Combining the two enzymes in the same reaction did not alter this result (lanes 11–12) suggesting that EcoP15I and PstII cannot interact to cut both strands of a DNA substrate. This differs from the situation observed with Type I enzymes (54) or with EcoP15I and EcoPI (37,56). On lambda phage DNA both PstII and EcoP15I produced a ladder of product fragments resulting from cleavage at multiple sites.

![Figure 7](https://academic.oup.com/nar/article/33/15/4775/2401036)
(lanes 1–6). Addition of AdoMet resulted in some inhibition due to co-methylation (lanes 4–6). However, combining EcoP15I and PstII actually generated more cleavage than either enzyme alone (compare lanes 2 and 3 to lane 5)—this is due to the combined cleavage of two independently acting systems. Therefore the inactivity on T7 cannot be due to non-specific inhibition of either PstII or EcoP15I by the partner enzyme. Instead, DNA cleavage by Type III enzymes must require a very specific interaction between the same (or closely related) proteins.

**DISCUSSION**

**PstII acts as a prototypical Type III enzyme**

As part of a general screening programme to search for new restriction modification systems, a segment of genomic DNA from *P. stuartii* 164 was cloned which carried a putative Type III RM system which has been named PstII [Results, Materials and Methods, (1)]. Our initial characterization of the enzyme activity of PstII indicated that it conformed to the prototypical Type III RM model as exemplified by EcoPI and EcoP15I. In fact, the recognition and cleavage sites of PstII show remarkable similarities to the EcoP15I sites (Figure 2A and Table 2). The differences in the Type IIIA and IIIB Res motifs [Figure 1, (33)] therefore, do not result in gross changes in enzyme mechanism. We did observe a relaxed specificity in the nucleotide hydrolysis of PstII that would reflect a looser arrangement of residues in the NTP binding site. Since the helicase motifs cluster in this pocket (33,57), it is possible that other Type IIIB enzymes will also utilize NTPs other than ATP. In the accompanying paper (15) we describe how disassembly of PstII by loss of a Res subunit can act to control relative modification/restriction activities. This appears to differ from EcoPI/EcoP15I where the tetramer is stable at nanomolar concentrations *in vitro* (34). It is possible that this difference may reflect alternative routes to prevent autorestriction by Type III enzymes [see Discussion (15)]. We did note that PstII was apparently not as freely transferable as EcoPI or EcoP15I suggesting that despite very similar enzyme activities, the control of those activities is different *in vivo*.

As we have observed previously for other Type III enzymes (36), AdoMet is not strictly required for DNA cleavage by PstII. Co-factor contamination was estimated as <15% with our preparations of PstII (data not shown), so it cannot be argued that every enzyme already has AdoMet bound. Instead we conclude that it is a general feature of Type III enzymes that, under certain conditions, AdoMet is not required as an allosteric activator. We did not observe any 2° cleavage events in the absence of the AdoMet. This may be because AdoMet does not have a ‘specificity factor’ role in PstII but perhaps a more likely explanation is that we could not add sufficient excess enzyme to induce the additional cleavage events. Given the dynamic assembly of PstII (15), this is a difficult question to pursue further.

How does PstII communicate between DNA distant sites? In the standard Type III model (2–4,47), communication is believed to be due to enzyme complexes bound at each site hydrolysing ATP and translocating towards each other along the intervening DNA—cleavage is activated when the enzymes collide. If this model is correct, and assuming enzyme collision occurs on average halfway between the sites, each PstII complex on pLJP11b would need to travel ~528 bp (one-half of the length of the DNA arc between the head-to-head sites, Figure 2B). With a maximum ATPase rate of ~40 ATP/site/min (Figure 6D), and given that production of the first strand break occurs within 10 s (Figure 5), it would appear that only ~6–7 ATP molecules will have been consumed by each PstII tetramer before cleavage is initiated. This corresponds to an apparent coupling of ~82 bp translocated for each ATP hydrolysed. This ‘step size’ is significantly larger than anything else measured for a DNA helicase or ATP-dependent DNA translocase (58–62). Either the Type III enzymes utilize their helicase motifs to move on DNA in a completely new and highly efficient manner, or they do not translocate at all.

In comparison to the Type I RM enzymes, our knowledge of the motor mechanism of the Type III enzymes is somewhat murky (33). There is clear evidence that simple DNA looping does not play a role (44). However, there has yet to be a direct measurement of a 1D linear translocation activity. It is proven that the Type I enzymes extrude a DNA loop during translocation ([13] and references therein) and on the basis of the shared SF2 helicase motifs, this model has also been adopted for the Type III RM system (47): there is evidence both for (63) and against (34,44,64) loop extrusion by Type III enzymes. In this study we have shown for the first time that the Type III communication requires specific interactions between distant enzymes (Figure 7). The glaring problem we now need to address is precisely how the Type III enzymes couple relatively low ATPase rates to communication events between distant DNA sites. The PstII enzyme will provide a valuable test system to compare with the EcoPI and EcoP15I enzymes.

**Table 2. Survey of strand bias of CANCAG sequences in representative bacteriophage genomes**

| Phage | CTGCTG (e.g. EcoP15I) | CTGATG (e.g. PstII) | CTGGTG | CTGTGT |
|-------|----------------------|---------------------|--------|--------|
| λ     | 41 31 43 22 15 9 12 | 11                  |        |        |
| T7    | 36 0                 | 34 0 35 1           | 0      | 23     |
| ΦA1122 | 42 1 29 0             | 32 4               | 1      | 20     |
| T5    | 65 45 29 35          | 40 28 38           | 38     | 38     |
| T3    | 48 6 30 86           | 41 47 36           | 46     |        |
| T4    | 57 58 50 86          | 41 47 36           | 46     |        |
| P1    | 34 32 37 10          | 67 59 28           | 33     |        |
| P2    | 17 25 41 17          | 10 12 10           | 11     | 11     |

A striking feature of the PstII recognition sequence is its similarity to that of the EcoP15I enzyme (Figure 2A): the sites share a common sequence motif, CANCAG. Although the Mod subunits of these enzymes have evolved to recognize a similar sequence, the assembly of the protein complex onto that site has not placed the catalytic residues at same relative positions—the locations of the methylated adenines and non-specific cleavage loci are quite different (Figure 2A). Given that both sequences show a strand bias in T7 phage DNA as a marker of Type III activity
DNA (55), we conducted a survey of strand bias in all CANCAG sequence in a number of different phage genomes (Table 1). In T7 and T7-related genomes, the CANCAG sequences show significant strand bias. This bias is not widespread amongst other phage however, although some strand bias of the PstI sequence is observed in T3. The more detailed statistical analysis of Krüger et al. (55) identified seven hexameric sequences which show significant strand bias in T7. The two sequences with the greatest statistical bias were CAGCAG (EcoP15I) and CATCAG (PstI). Therefore, this earlier study had already predicted the existence of PstI. In a similar manner, all of the palindromic sequences identified as completely absent from T7 20 years ago (55) can now be assigned to a corresponding Type II activity (1). Sequence asymmetry in the T7 genome therefore acts as an historical imprint of restriction endonuclease activity (56).

The other biased hexameric T7 sequences identified by Krüger et al. (55) as statistically significant were CATAGC, CAGAGC, GAGCTT, GCACTT, GCAGAG and CATAGC. For which strand there are a large number of sequences which are not completely absent from one or other strand but for which strand bias is still significant, such as CAGCAG (Table 2). With the discovery of a second Type III sequence which shows strand bias in T7 it is possible that selective pressure from additional, as yet unidentified, Type III enzymes has resulted in strand bias in many different sequences. Sequence asymmetry in genomes is a recognized feature of evolution in all organisms (65,66) but is particularly marked in T7 (67). Although the spontaneous mutational spectra of many phage should be similar, it seems that T7 has been particularly successful in harnessing evolutionary drift to prevent cleavage by both Type II and III restriction enzymes. To further safeguard against restriction by Type I enzymes, the antirestriction ocr gene 0.3 of T7 is the first to be expressed during the early stages of internalization (68).

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