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Purification and Characterization of New Type II Restriction Endonucleases

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Abstract

Twelve bacterial strains were screened for the presence of new type II restriction endonucleases. Two are reported positive for the enzyme activity, and designated as *Bth*DI, *Sar*I, from *Bacillus thuringiensis* D4 (CAMB 2657) and *Streptomyces aureomonopodiales* (ATCC 19825). These enzymes have been purified by a combination of gel filtration, ion exchange and affinity chromatography. The DNA sequences recognized by these enzymes have been determined by analysis of the fragment patterns generated on different substrate DNAs, Lambda, T7, pUC19, pBR322, PhiX174RFI, and Adenovirus-2 (Ad-2). The cleavage sites within the recognition sequences have also been established by primed synthesis. Accordingly, the newly discovered enzymes *Bth*DI and *Sar*I recognize and cleave 4-6 nucleotide long stretch of DNA, CC↓ AGG, AGG↓ CCT, respectively.

Introduction

Restriction-modification (RM) systems have been identified in about 25 per cent of the presently examined prokaryotes (Wilson and Murray, 1991). Restriction-modification system comprises two components, restriction endonuclease, and and its cognate methylase. Restriction enzymes of the host restriction modification systems destroys any foreign DNA, whereas, methylase catalyzes the transfer of methyl group from S-adenosylmethionine (AdoMet) to adenine or cytosine residue and thus protect cells DNA from restriction enzyme cleavage.

All known restriction enzymes have been grouped into three classes, type I, II, III on the basis of their subunit structure, cofactor requirements, substrate specificity and several other features (Yuan, 1981). Recently, two new types of restriction endonucleases have been discovered which are different from previously known three categories of endonucleases. These have been named as type IV and V (Janulaitis *et al.*, 1992; Petrusyte *et al.*, 1987).

Type II restriction enzymes are strain specific and are regarded as the simplest, requiring Mg^{++} for their catalytic function. The majority of enzymes, so far characterized recognize 4-8 nucleotides long DNA sequences and cleave double stranded DNA within or close to the recognition sequence. Enzymes isolated from quite different organisms may recognize the same DNA sequence and are known as isoschizomers. However, they may be functionally distinguished from each other on the basis of site of cleavage by the endonuclease, the sensitivity of the cleavage reaction to DNA modifications and the site of methylation by the methylase. The type II restriction endonucleases have been extremely useful in recombinant DNA work (gene mapping, physical mapping of DNA molecules, gene cloning, DNA sequencing, and construction of new genomes). These enzymes exhibit remarkable diversity. A total of 2,821 restriction enzymes are known, including 232 different type II specificities, (Roberts and Macelis, 1997). On theoretical consideration it can be assumed that the enzyme specificity is not exhausted. In fact there are many more potential sites on prokaryotic and eukaryotic DNA, for which specific enzymes must exist. This paper reports the purification and characterization of two new type II restriction endonucleases that recognize and cleave 4-6 nucleotide long stretch of DNA. The enzymes recognize previously known specificities, however, their use as laboratory reagents offers convenience in preference to the previously known enzymes.

Materials and Methods

Enzymes and chemical reagents: Type II restriction enzymes and *E. coli* DNA polymerase (Klenow) were obtained from New England Biolabs, (NEB). Sequenase version 2.0 sequencing kit was purchased from United States Biochemicals (USB), [35S]dATP (1000- 1300 Ci/mmole) was purchased from New England Nuclear Corporation (NEN) and Amersham. Biogel A-0.5M was from Bio-Rad company. DEAE-cellulose and Phosphocellulose were from Sigma. Heparin agarose was obtained from Pharmacia LKB Ltd. All other organic and inorganic chemicals were obtained from either Sigma Chemical company or E. Merck.

Bacterial strains and media: Twelve bacterial strains were collected from different ecological environments of Pakistan and taxonomically characterized according to Bergey's Manual (Buchanan *et al.*, 1974). Some of the strains were obtained from American Type Culture Collection (ATCC). *E. coli* JM103 and JM107 were obtained through the courtesy of J. Messing, University of Minnesota. T7 phages and *E. coli* BL21 was obtained from F.W. Studier, BrookHaven National Lab, Uptown NY. GM272 and GM1519 (CI 857 *Sam*7 lambda lysogen in GM119) were obtained from M.G. Marinus, University of Massachusetts, Worcester. *E.coli* RRI strain was obtained from Cold Spring Harbor Laboratory, NY.

For M13 phage infection and template preparation, JM103 or JM107 were maintained on Minimal plates and grown on 2YT medium (Miller, 1972). *Bacillus thuringiensis* D4(CAMB 2657) was grown on Brain Heart Infusion.

Streptomyces aureomonopodiales (ATCC 19825) was grown on Keith's STP broth (0.8 per cent Nutrient broth, 34 per cent Sucrose, 1 per cent MgCl2 pH 6.0, 0.5 per cent Glucose), and maintained on ISP-2 plates (0.4 per cent Yeast extract, 1per cent Malt extract, 0.4 per cent Bactodextrose, pH 7.3, 2 per cent Agar).

All substrate DNAs except Adenovirus-2 and PhiX174RFI used in the study were prepared at CEMB using standard protocols. Lambda DNA was prepared from GM1519 Lambda lysogen as described (Maniatis *et al.*, 1982). Plasmid DNAs (pBR322, pUC19) were prepared from *E.coli* strains, GM272 and RRI, respectively by alkaline extraction method (Birnboim and Doly, 1979). T7 phage DNA was prepared from *E.coli* BL21 strain infected with T7 phages at 3-5 moi. T7 phages were purified as described (Dunn and Studier, 1983). Adenovirus-2 and PhiX174RFI were a gift from Dr. R. J. Roberts, New England Biolabs, Beverly, MA. pDCM23 was given by Dr. Anjum Sohail, CEMB. Genomic DNAs of strains were prepared as described (Silhavy *et al.*, 1984).

Screening of bacterial strains for type II restriction enzymes: Bacterial cells were cultured in 100ml-1L of appropriate growth media at 28-37°C. Cells were lyzed by lyzozyme treatment and ultrasonication to prepare crude protein extract. 1-2ug lambda and T7 phage DNAs were digested with 2-15ul crude protein in 50ul reaction volume containing 1X reaction buffer (10mM Tris-HCl pH 7.5, 10mM MgCl2, 10mM 2-Mercaptoethanol) at 30-37°C in two sets of reactions. One was incubated for 10-15 min and the other was incubated for 30-45 min. Reactions were stopped with 1/6 volume of loading dye and electrophoresed on 0.7-1.0 per cent agarose gels. Some strains were screened by purifying the crude protein extracts through Biogel A-0.5M (Gel filtration).

Isolation and purification of restriction enzymes: Cells Were grown under forced aeration in a fermenter containing an appropriate media and temperature till late log phase (Table 1) and harvested by centrifugation. Crude cell extracts were prepared by lyzozyme treatment and ultrasonication in lysis buffer (10mM Tris-Cl pH 7.5, 10mM 2-Mercaptoethanol). Nucleic acids were removed by gel filtration or streptomycin sulfate treatment at 0.8 per cent final concentration. Restriction endonucleases were purified from exonucleases through ion exchange (DEAE cellulose, Phosphocellulose) and affinity chromatography (Heparin agarose), concentrated by ultrafiltration and ultimately stored in 50 per cent glycerol at -20°C. To check the enzyme activity, assays were performed according to standard protocol (Riazuddin *et al.*, 1987; Sohail *et al.*, 1987).

Characterization of restriction enzymes: Different DNA substrates, Lambda phage, T7 phage, pUC19, PhiX174RFI, pBR322, Adeno-2, and were used to characterize newly isolated enzymes in appropriate reaction buffers. Samples wre electrophoresed on 0.7 per centagarose gels. Resulting fragmentation patterns were compared with computer generated digests of prototype restriction enzymes on different substrate DNAs. In case of resemblance, with a previously known restriction enzyme, substrate DNAs were digested in the presence of both the newly discovered and the previously known enzymes. Number of cleavage sites on different DNA substrates for the new enzymes and their isoschizomers was determined. The identity of recognition sequence and cleavage site was confirmed by DNA sequence analysis using adenovirus-2 library in M13 phage provided by Dr. R. J. Roberts, New England Biolabs Inc, USA.

M13 phage having the required restriction enzyme site close to (not more than 200bp away) 17mer M13 primer (-40) was searched from the Ad-2 library and used to make template DNA. Sequencing of template DNAs was performed with a Sequenase version 2.0 kit (USB) using dideoxy chain termination method (Sanger *et al.*, 1977). Cleavage site for the enzymes were determined by primed synthesis reaction (Brown and Smith, 1980). All the sequencing and cut-repair reactions were loaded on 6 per cent polyacrylamide gels. Gels were electrophoresed in 1X TBE (0.089M Tris, 0.089M Boric acid, 0.002M EDTA), fixed in 10 per centmethanol and 10 per centacetic acid for 15-20 min, dried under vacuum and were finally exposed to X-ray films (Kodak or Fuji). Autoradiograms were developed.

The newly isolated restriction enzymes were checked for their sensitivity to heat, and compared with their isoschizomers. The enzyme was incubated at 65-70°C for 20, 30, 45 minutes. Varying amounts of the heated enzyme were added to reaction mixtures containing the appropriate buffer, 1.0ug substrate DNA, and incubated at 37°C for 60 minutes to see any digestion (complete or partial).

To check the modification profile of DNA and sensitivity of the restriction enzyme to that modification, genomic DNA of the host strains were isolated. 1-2ug of each DNA was incubated with its own restriction enzyme and the isoschizomer enzyme for 1-2h at 30-37°C. Cellular DNA was also digested with *Eco*RII/*Bst*NI pair to test for the presence or deficiency of dcm like methylation. Methylated lambda (*dam*$^+$ *dcm*$^+$) DNA or pDCM23 (containing dcm methylase gene from *E. coli* K-12 as a *Bam*HI-*Sal*I 1.8 fragment in pBR322) with modified *dcm* sites was also used as substrate to check methylation sensitivity by comparing with methylation sensitivities of some of the restriction enzymes already reported (McClelland *et al.*, 1994).

Results

Purification and characterization of *Bth*DI: *Bacillus thuringiensis* D4 (CAMB 2657), source strain of *Bth*DI enzyme, has a low background of exonucleases and gives a good yield of cells 6-7g/l in BHI at 30°C (Table 1).

Table 1: Properties of bacterial strains positive for type II restriction endonucleases.

Bacterial Strains	CAMB No.	Source	Growth Media	Growth Temperature	Cell Yield g/litre
Bacillus thuringiensis D4	2657	Soil	BHI	30°C	6-7
Streptomyces aureomonopodiales	4004	ATCC 19825	Keith's STP	30°C	13-14

Table 2: Number of cleavage sites on different DNA substrates.

Enzyme	pUC19	Lambda	Adeno-2	X174	pBR322	T7
BthDi	5	71	130	2	6	2
(BstNI)	0	6	11	1	0	1

enzyme was purified by chromatography through Biogel A-0.5M DEAE-cellulose and Phosphocellulose (PC). BthDI eluted at 0.10-0.18M KCl from DEAE-cellulose and at 0.32-0.48M KCl from Phosphocellulose column (Table 3). The enzyme was free of all contaminating nuclease activity as checked in a 16h overdigestion reaction. The enzyme acts optimally at 150mM NaCl, 10mM Tris-Cl pH 7.5, 10mM $MgCl_2$, 1mM DTT at 37°C. The size and number of fragments produced by *Bth*DI digests on lambda, T7, pBR322, pUC19 DNAs match exactly with those of *Bst*NI that recognizes 5'-CCWGG-3' sequence.

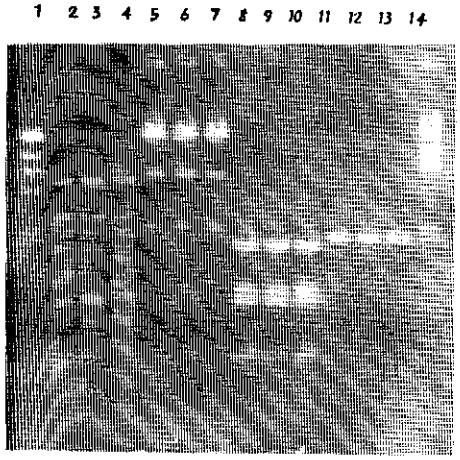

Fig. 1: Characterization of BthDI by double digestions on 0.7 per cent agarose gel. Lanes 1 and 14 contain lambda DNA digested with HindIII as marker, Lanes 2, 5, 8 and 11 contain lambda, T7, pBR322, pUC19 digested with *Bst* NI. Lanes 3, 6, 9, 12 contain above DNAs digested with *Bth*DI and *Bst*NI> Lanes 4, 7, 10, 13 contain same DNAs digested with *Bth*DI.

Number of cleavage sites given in Table 2. This was further confirmed by double digest analysis using *Bth*DI and *Bst*NI enzymes (Fig. 1). Its isoschizomer *Bst*NI exhibits optimal activity at 55-60oC in 50mM NaCl and requires the presence of 100ug/ml BSA (Schildkraut *et al.*, Unpublished observations, NEB catalog, 1994). Moreover, *Bst*NI has special storage buffer as compared to *Bth*DI, which can be stored simply in 50 per centglycerol at -20°C. *Bth*DI and *Bst*NI enzymes were checked for their heat inactivation property. 20 units of *Bst*NI remained active after heating at 65°C, but 10 units of *Bth*DI were inactivated at 65°C (Table 3). This property can be exploited in multiple enzyme digestions.

M13 phages # 1553 containing *Bth*DI recognition stie 136bp away from 17mer M13 primer(-40) was selected from Adenovirus-2 library in M13 for sequencing and cleavage stie determination. The resulting radiolabelled cleavage product was electrophoresed on 6 per cent polyacrylamide gel containing 7M urea along with the DNA nucleotide sequence from the same phage DNA. Sequencing data of *Bth*DI and its isoschizomers *Bst*NI and *Eco*RII comfirms that the radiolabelled cleavage product generated by *Bth*DI resembles *Bst*NI but differs from that of *Eco*RII. i.e. the enzyme cleaves after second C rather than the first C in the recognition sequence, 5'-CC↓WGG-3'(Fig. 2, Table 3).

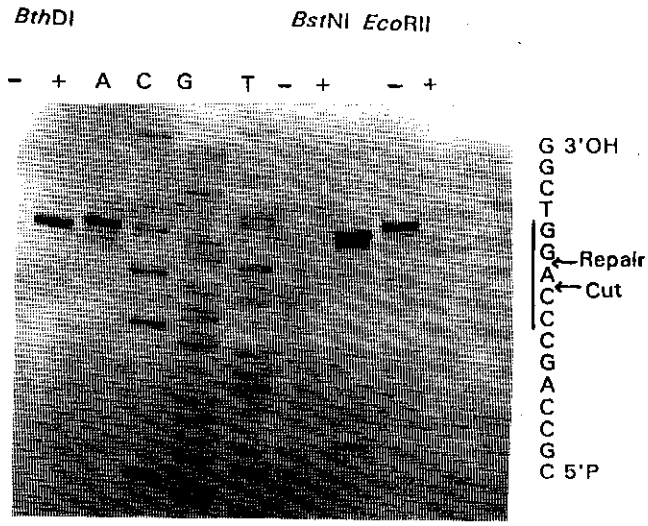

Fig. 2: Cleavage position of *Bth*DI in comparison with *Bst*NI and *Eco*RII on 6 per cent polyacrylamide gel. Autoradiogram shows sequencing reactions A, C, G, T and cleavage sites for *Bth*DI, *Bst*NI and *Eco*RII usig Adeno-2 template DNA in lanes (—), The cleavage products treated with Klenow are in lanes (+). Recognition sequence shown by a line.

Lambda (*dcm*$^+$ *dam*$^+$) and pDCM23 DNA with 5-methyl

Table 3. Properties of newly isolated enzymes.

Enzyme/ Isoschizomer	Column Media	Elution profile during chromatographic separation: Salt conc. of the eluate	Salt Conc. for optimal activity	Recognation seq./cut site	Heat inactivation at 65-70°C
*Bth*DI (BstNI)	Biogel A-0.5M DEAE-cellulose phosphocellulose	Fraction # 19-47 (145ml) 0.10-0.18 M KCL	150mM Nacl	CC↓AGG	100% Inactivation
SarI (StuI)	Biogel A-0.5M DEAE-cellulose Phosphocellulose heparin agarose	Fraction # 30-42 (65ml) 0.40-0.50 M KCL wash 0.34-0.54M Nacl	50mM Nacl 20mM Kcl	AGG↓CCT	100% Inactivation

↓ Cut site within the recognition sequence.

modification on internal C in the recognition sequence Cm5CWGG, were digested with *Bth*DI and *Bst*NI enzymes, but remained uncut with *Eco*RII. It has already been reported (McClelland *et al.*, 1994) that *Bst*NI can cut 5-meC modified DNA at internal, external or both cytosines in recognition sequence CCWGG. Since, *Bth*DI can cleave methylated DNA at internal cytosine in Cm5CWGG, like *Bst*NI, it is not sensitive to 5-methyl modification at the internal cytosine. However, its sensitivity to other possible methyl modifications was not checked (data not shown).

Purification and characterization of *SarI*: SarI has been isolated from *Streptomyces aureomonopodiales* (ATCC 19825), a plasmid bearing strain. Since *Streptomyces* are highly aerobic, cultures were shaken vigorously for good aeration. Organisms grew in the form of mycelial pellets (aggregates of spores), resulting in a high yield of cells 13-14g/l in Keith's STP growth medium at 30°C (Table 1). Due to spore formation, cells were not easily lyzed with lysozyme at 4°C. So these were incubated with lysozyme at 30°C followed by extensive ultrasonication. *Streptomyces aureomonopodiales* has a low background of non specific nucleases which can be removed completely by the purification scheme given (Table 3) after giving 0.8 per centstreptomycin sulfate and 70 per cent ammonium sulfate treatment. The enzyme eluted at 0.40-0.50M KCl from DEAE-cellulose. It did not bind to phosphocellulose, and was collected in column wash. The enzyme eluted at 0.34-0.54M NaCl from Heparin agarose column (Table 3). Stul has also been purified from gel filtration and ion exchange columns after giving 2 per cent streptomycin sulfate, and 40-80 per cent ammonium sulfate treatment. However, no affinity column was used in this case (Shimotsu *et al.*, 1980). *SarI* enzyme activity was checked in different reaction buffers and the optimal conditions for enzyme activity are 20mM KCl as well as 50mM NaCl, in 10mM Tris-Cl in pH 7.5, 10mM MgCl2, 1mM DTT at 37°C (Table 3). Both *SarI* and *StuI* (20 units) were inactivated at 65°C, when checked for their sensitivity to heat (Table 3). It was also found that *SarI* remained fully active upto 45°C for 20 minutes.

The recognition sequence 5'-AGG↓CCT-3' was confirmed by single and double digests of lambda phage, Adeno-2, PhiX174RFI and pUC19 DNAs with *SarI* and *StuI* (Fig. 3). The number of cleavage sites on these substrate DNAs are given in Table 2. M13 phages # 1144 containing *SarI* recognition site 123bp away from M13 primer was selected from Adenovirus-2 library in M13 for determination of cleavage site for *SarI*. The results show that *SarI* cleaves the phosphodiester bond between the G and C residues in the recognition sequence, 5`-AGG↓CCT-3`, producing blunt end fragments (Fig. 4).

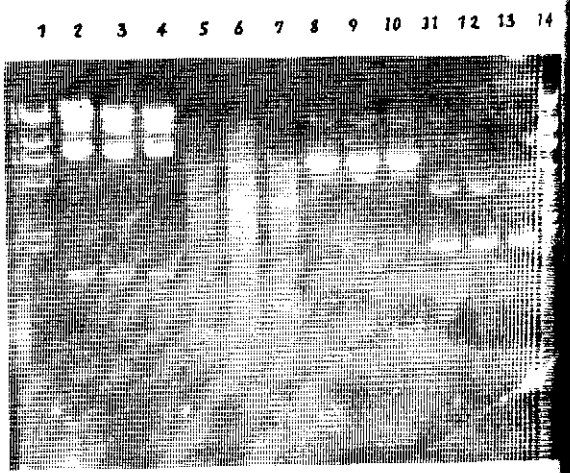

Fig. 3: Characterization of *SarI* by double digestions o 0.7 per cent agarose gel. Lanes 2, 5, 8 and 1 show single digests of lambda, Adeno-2,PhiX17 and pUC19 with *SarI*. Lanes 3, 6, 9, 12 sho double digests of above DNAs with *SarI* and Stu Lanes 4, 7, 1, 14 show lamba *Hind*III size mark

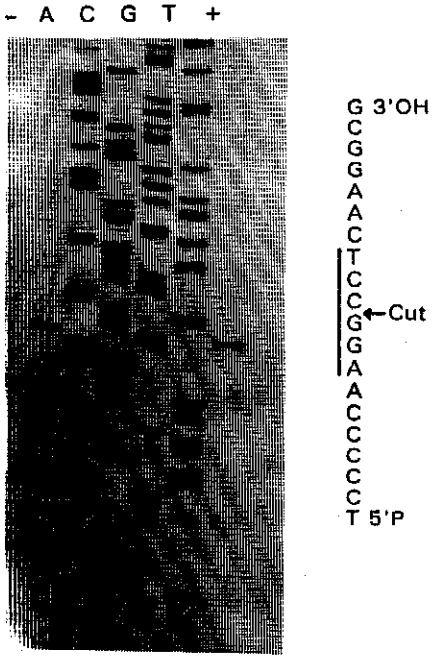

Fig. 4: Cleavage position of *SarI* on 6 per cent polyacrylamide gel. The cleavage product of *SarI* is in lane (—) and the same cleabage product treated with KIwnow is in lane (+). Sequencing reactions are in lanes A, C, G, T. Recognition sequence shown by a line.

Cellular DNA from *Streptomyces aureomonopodiales,* the source strain of *SarI,* was resistant to *StuI* cleavage. The prototype enzyme, *StuI* is sensitive to N4-methyl modification on second cytosine or 5-methyl modification on first or second cytosine in the sequence AGG CCT (McClelland *et al.*, 1994). So, genomic DNA from *Streptomyces aureomonopodiales* may have any one of these modifications and *SarI* is sensitive to that modification (data not shown). On the basis of these results, it is concluded that *SarI* is a true isoschizomer of *StuI* (Shimotsu *et al.*, 1980).

Discussion

Since the discovery, of first site specific restriction endonuclease (Smith and Wilcox, 1970), efforts have continued to search for new restriction enzymes to improve the precision of recombinant DNA work. During the present study twelve bacterial strains available from ATCC and CEMB culture collection were screened for the presence of type II restriction enzyme activity. Of these strains, two were found positive. This shows that one in six of all bacteria examined appear to have only one type II restriction enzyme. No isolate had two or more enzymes. These isolates have simple growth media with an optimum growth temperature at 30°C.

The newly isolated enzymes have been characterized. They are found to be the isoschizomers of their prototype enzymes. These enzymes are easy to purify, have simple reaction and storage conditions and are optimally active at 30-37°C. Incubation at 65-70°C for 20 minutes inactivates all the enzymes, which is a good property for multiple enzyme digestion reactions. The two enzymes recognize 4-6 nucleotide long DNA sequences which are symmetric and palindromic. The nature of the cut site and the number of cleavage sites on different DNAs is comparable to the prototype enzymes. DNAs from the host strains were prepared and digested with a variety of restriction enzymes to check the modification of the DNAs as described (Brooks and Roberts, 1982). From the experiments performed, it was concluded that DNA of a restriction enzyme strain was not cleaved by its own enzyme and it was also resistant to its isoschizomer with same specificity. The methylation sensitivity of new enzymes is also comparable to those of the prototype enzymes already reported (McClelland *et al.*, 1994).

Since, most of their properties are comparable to those of the previously known enzymes, these can replace the commercially available prototype enzymes to fulfil our own experimental needs. Despite a large number of restriction enzymes already known, many more specific enzymes must exist in nature. Therefore, screening microbes for presence of novel type II restriction enzymes may be continued in future.

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