A thermostable, sequence-specific restriction endonuclease from *Bacillus stearothermophilus*: BstPI

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ABSTRACT

A restriction endonuclease, BstPI, was purified from a strain of *B. stearothermophilus*, and its cleavage specificity was determined. The enzyme cleaves at palindromic sites of the general structure:

5' -G-G-T-N-A-C-C- 3'

3' -C-C-A-N-T-G-G- 5'

where N·N' can be any base pair. It produces phosphorylated 5'-termini which are single stranded over a length of 5 nucleotides. Ends generated by cleavage with BstPI can be rejoined by DNA ligase.

INTRODUCTION

Several strains of *B. stearothermophilus* have been shown to contain restriction endonucleases (1,2). We have purified and characterized a restriction enzyme of class II from *B. stearothermophilus* strain ATCC 12980, BstPI, and have determined that it recognizes the palindromic heptanucleotide sequence 5'-G-G-T-N-A-C-C-3' 3'-C-C-A-N-T-G-G-5' and introduces staggered endonucleolytic cleavages between the two G residues of that sequence.

MATERIALS AND METHODS

Seed cultures of *Bacillus stearothermophilus* strain 12980 were purchased from the American Type Culture Collection (Rockville, Md.) and stored in ampoules at 4°C.

DNA: Plasmid pCRI DNA and bacteriophage Ø29 DNA were prepared by W. Boll of this Institute. Bacteriophage PM2 DNA was a gift from Dr. Th. Koller (Institut für Zellbiologie, ETH, Zürich). Plasmid pSF2124 DNA was a gift from S. Zimmer (University of Basel, Switzerland), plasmid PßG DNA (3) and bacteriophage λ C1857S7 DNA (4) were prepared according to described procedures.

Three hybrid plasmids containing DNA complementary to bacteriophage Q β RNA were kindly supplied by Dr. Billeter of this Institute:

a) pQ β 160 (full designation: Z-pCRI(EcoRI)/Q β partial (dAdT)-160) is a pCRI plasmid carrying about 900 nucleotides from the 5'-terminal part of the Q β genome inserted into the EcoRI site (5,6).

b) pQ β 32 (full designation: Z-pCRI(EcoRI)/Q β inf (dAdT)-32) contains the full sequence of the Q β genome inserted in the EcoRI site of plasmid pCRI and gives rise to viable Q β phage (7).

c) pQ β HBR (full designation: Z-pBR322(HindIII)/Q β inf(HindIII)-1) is a pBR322 derivative containing a complete DNA copy of the bacteriophage Q β genome inserted into the HindIII site. It too gives rise to viable Q β phage (8).

Restriction endonucleases: EcoRI was purchased from the Microbiological Research Establishment, Porton (U.K.) and XhoI was purchased from New England Biolabs. BspI was a gift from Dr. A. Kiss (University of Szeged, Hungary) and BamHI was a gift from M. Kappeler of this Institute.

Other enzymes: Bacterial alkaline phosphatase was obtained from Worthington Biochemical Corp. and calf intestine alkaline phosphatase was from Boehringer, Mannheim (Germany). Polynucleotide kinase (from T₄-infected E.coli) was obtained from P-L Biochemicals Inc. and DNA ligase (from T₄-infected E.coli) was a gift from Dr. K. Murray, University of Edinburgh (U.K.). Pancreatic DNase I and snake venom phosphodiesterase were obtained from Worthington.

Nucleotides: The dinucleotides dpGpG and dpGpT (ammonium salts) were purchased from P-L Biochemicals Inc. γ -³²P ATP was obtained from NEN.

Growth of cells: 3 x 500 ml of a 3.7% brain-heart infusion medium in Erlenmeyer flasks were inoculated with an overnight culture of *B. stearothermophilus*. The cells were grown at 55°C under agitation in a rotary shaker (140 rpm) to late log phase (apparent OD₅₄₆ = 1.55). Cells were harvested by centrifugation. The wet weight of cells recovered was 3.2 g.

Purification of enzyme: The enzyme was isolated according to the method of Bickle et al. (9) using a phosphocellulose column

(10 ml) instead of heparin-agarose. Elution was by a linear gradient of 0.1 M - 0.6 M NaCl in 50 mM Tris-HCl (pH 7.5), 0.05 mM DTT at a flow rate of 0.1 ml/min and a fraction size of 2.5 ml. Endonuclease BstPI eluted at 0.18 M NaCl. The active fractions, 7.5 ml, were pooled and dialyzed against a mixture containing 70% glycerol and 30% 10 mM Tris-HCl (pH 7.5) until the volume was reduced to 1.8 ml. Bovine serum albumin (BSA) was added to a final concentration of 100 µg/ml in order to stabilize the enzyme. Endonuclease BstPI could be stored for at least 8 months at -20°C without loss of activity.

Assay of enzyme: 1 µg of DNA (pQβ160) was incubated with enzyme and restriction buffer (90 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂) at 37°C for 1 h. The reaction was stopped by adding a mixture of glycerol, agarose, EDTA and bromophenol blue (10). The DNA products were separated by agarose gel electrophoresis (see below) and the extent of cleavage was estimated visually on the photographs by comparison with a total pQβ160 DNA digest. 1 unit of enzyme is the amount necessary to digest 1 µg of pQβ160 DNA in 1 h at 37°C.

Agarose gel electrophoresis: Restriction fragments were separated on 1% agarose slab gels in a buffer containing 36 mM Tris-HCl (pH 7.9), 30 mM sodium phosphate (11) or 50 mM Tris-HCl (pH 7.8), 20 mM sodium acetate. Gels were stained in 0.5 µg/ml ethidium bromide and photographed under ultraviolet illumination.

Terminal labeling and sequencing: Restriction fragments were treated with bacterial or calf intestine alkaline phosphatase and labeled at the 5' ends with γ-³²P ATP and polynucleotide kinase (12). After secondary cleavage the labeled fragments were separated by electrophoresis on 5% polyacrylamide gels in Tris-phosphate buffer (11). Sequencing of DNA was performed according to the Maxam-Gilbert technique (12).

Assay for phosphatase and exonuclease contamination: 0.04 µg 5'-³²P-labeled restriction fragments were incubated with 0.5 µl (1 unit) of endonuclease BstPI under the assay conditions described above. The digest was subjected to paper electrophoresis on Whatman 3MM paper in pyridine-acetate buffer (pH 3.5, ref. 13). Released radioactivity was determined by autoradiography and scintillation counting of the labeled spots.

RESULTS

a) Isolation, partial purification and general properties of the enzyme.

Bacillus stearothermophilus ATCC 12980 was grown, harvested and lysed by sonication as described in the Materials and Methods section. After removal of cell debris by centrifugation, the supernatant was assayed for endonuclease activity on plasmid pQ β 160 or on phage lambda DNA. The extract obtained from 1 g of cells was found to contain approximately 7500 units of an endonuclease activity cleaving pQ β 160 DNA into four fragments. The activity was purified by fractionation with polyethylenimine and ammonium sulfate (9) and chromatography on phosphocellulose as detailed in the Materials and Methods section. The specific activities and yields obtained in the purification steps are presented in Table 1.

At this stage of purification the preparation was virtually free of contaminating exonucleases and phosphatases as demonstrated by incubation of 5'-³²P-labeled restriction fragments with a 25-fold excess of enzyme. This treatment caused the release of less than 5% of the terminal radioactivity as inorganic phosphate or mononucleotide.

The BstPI-catalyzed DNA cleavage reaction was found to proceed optimally in 90 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl₂. Increasing the MgCl₂ concentration up to 15 mM had little effect, but lowering it or the ionic strength resulted in redu-

TABLE 1 Partial purification of restriction endonuclease BstPI.

Enzyme preparation	Total activity (units)	Spec. activity (U/mg protein)	Vol. (ml)	Total protein (mg)
Crude supernatant	24'000	70	8.0	346
PEI precipitation	19'500	110	6.5	177
(NH ₄) ₂ SO ₄ precipitation	17'400	130	5.8	136
PC-column	3'600	3600	7.5	1

The starting material was 3.2 g (wet weight) of *B. stearothermophilus*, obtained from a 1500 ml culture. Details of the enzyme preparation are given in the Materials and Methods section.

ced reaction rates. Not surprisingly, the enzyme showed a high thermal stability. Its specific activity at 55°C was about 25% higher than at 37°C and it remained unchanged up to 65°C, the highest temperature tested.

b) Cleavage patterns of various DNA substrates.

A number of viral and plasmid DNA preparations were digested with restriction endonuclease BstPI and analyzed by electrophoresis on 1% agarose gels. Results are shown in Fig. 1 and Table 2. An endonuclease EcoRI digest of phage lambda DNA is included in Fig. 1 (lane a) for comparison. Phage PM2 DNA (Fig. 1, lanes

Figure 1. Restriction fragment patterns of various viral and plasmid DNAs obtained by cleavage with endonuclease BstPI. Reaction mixtures (50 μ l) containing DNA (1.25 to 5 μ g), restriction buffer, and restriction endonuclease (2 units/ μ g DNA) were incubated at 37°C for 1 h. Portions containing 0.25 to 0.5 μ g of DNA were separated by electrophoresis on 1% agarose slab gels in Tris-acetate (pH 7.8) buffer as described in the Methods section. a and b, bacteriophage λ DNA; c, pSF2124 DNA; d, pQ β HBR DNA; e, pCRI DNA; f and g, P β G DNA; h and i, bacteriophage PM2 DNA. All samples were treated with endonuclease BstPI except sample a, which was treated with endonuclease EcoRI, and samples f and h, which were incubated without enzyme. Samples containing λ DNA were heated to 75°C for 5 min prior to gel electrophoresis.

TABLE 2 DNA fragments obtained from various viral and plasmid DNAs by restriction with endonuclease BstPI.

DNA	Minimal number of cleavage sites	Approx. size of fragments (kb)
λ C1857S7	11	8.3; 6.7; 5.9; 5.4; 4.5; 4.1; 3.4; 2.3; 2.0; 1.4; 1.3; 0.8
ϕ 29	2	11.5; 6.0
pQ β HBR	3	6.5; 1.859*; 0.553*
P β G	1	5.7 (full size linear DNA)
pCRI	2	11.5; 1.3
pSF2124	4	4.9; 1.7; 1.6; 1.1
ϕ X174 fd PM2 SV40 pBR322	no cleavage	

Reactions were carried out and products were analyzed as indicated in the legend to Fig. 1 and in the Methods section. Fragment sizes were estimated by calibration against endonuclease EcoRI fragments of λ C1857S7 DNA (21) and against two endonuclease BstPI fragments (marked *) of plasmid pQ β HBR whose size was known from DNA sequencing (M.A. Billeter, personal communication).

h,i) is not cleaved by endonuclease BstPI. By a treatment sufficient to cleave the other DNAs shown, only a trace amount of PM2 DNA is converted from the supercoiled to the relaxed form, indicating a very low level of non-specific nicking activity present in the enzyme preparation.

c) Determination of the DNA sequence recognized by endonuclease BstPI.

Most of the work leading to the elucidation of the specificity of restriction endonuclease BstPI was carried out on plasmid pQ β HBR, a recombinant plasmid consisting of the vector pBR322 and a complete DNA copy of the RNA genome of coliphage Q β (8). This plasmid is cleaved by endonuclease BstPI at 3 sites (Fig. 1, sample d), which are located in the Q β specific portion. Fig. 2 shows a partial restriction map of the Q β sequence insert (ref. 14, and Ph. Mekler and M. Billeter, personal communication).

In order to determine the nucleotide sequence at cleavage site B, plasmid pQ β HBR DNA was cleaved with restriction endonuc-

Figure 2. Partial restriction map of the Q β sequence insert of plasmid pQ β HBR.

The data are from ref. 14 and Ph. Mekler and M.A. Billetter (personal communication). As explained in the text, a XhoI-EcoRI fragment (drawn in enlarged scale) of plasmid pQ β HBR, labeled at the XhoI site, was used to determine the nucleotide sequence across the BstPI(B) site. The XhoI-BstPI fragment (51 bp, also labeled at the XhoI site) served as a marker to indicate the position of cleavage by endonuclease BstPI (see text and Fig. 3). *, 32 P label.

lease XhoI, treated with phosphatase and terminally labeled with polynucleotide kinase and γ - 32 P ATP. A portion of the labeled DNA was cleaved by restriction endonuclease EcoRI and the fragment containing the BstPI(B) site was isolated by polyacrylamide gel electrophoresis. It was sequenced according to Maxam and Gilbert (12). Another portion of the labeled DNA was cleaved by BstPI and coelectrophoresed on the sequencing gel to provide a marker allowing to identify the location of the cleaved inter-nucleotide bond.

The pattern obtained by autoradiography of the gel is shown in Fig. 3. Clearly, the XhoI-BstPI fragment (51 nucleotides) has the same electrophoretic mobility as the fragment resulting from cleavage following the second G residue, (i.e. giving rise to the third G band) of the sequence 5'-AGGTTACCGC 3'. This sequence contains the palindromic element GGTNACC. The same palindromic sequence is also found at the locations where the two other BstPI sites of the Q β genome have been mapped: Site A about 20 nucleotides from the 5' end, site C about 600 nucleotides to the right of the first BamHI site, approximately 2400 nucleotides from the 5' end (14).

Fig. 4 shows the nucleotide sequences around sites A, B and C; the sequence at site A was known from earlier RNA sequencing studies (15), the sequences near all sites have recently been determined on plasmid pQ β 32 (ref. 16, and M. Billeter, personal communication). Comparison of the three regions shows that their only common feature consists of the palindromic elements mentioned above, the middle and the flanking positions can obviously be occupied by any nucleotide. These sites are located at the nucleotide positions 19-25 (A), 572-578 (B) and 2431-37 (C) from the 5' end of the Q β genome (M. Billeter, personal communication).

In order to prove that all three of these sequences are actually cleaved by endonuclease BstPI, the mixture of the three BstPI fragments obtained from plasmid pQ β HBR was treated with phosphatase and terminally labeled with polynucleotide kinase and γ -³²P ATP. After further fragmentation with restriction endonuclease TaqI six labeled DNA fragments could be resolved by polyacrylamide gel electrophoresis, each containing one of the labeled 5' ends (not shown). The nucleotide sequences adjacent to the labeled termini were determined by the Maxam-Gilbert method (12). The sequences found could be identified with those previously determined at the palindromic sites A, B and C; they are indicated in Fig. 4 by arrows. On the basis of this and the previous experiment, we conclude that the interrupted palindromic

Figure 3. Determination of the nucleotide sequence near the BstPI site B of pQ β HBR DNA. Autoradiograph of Maxam-Gilbert sequencing gel obtained from the XhoI-EcoRI (300 bp) fragment of pQ β HBR terminally labeled at the XhoI site (see text and Fig. 2). Plasmid pQ β HBR DNA was cleaved with endonuclease XhoI, the fragments were treated with calf intestine alkaline phosphatase and terminally labeled with polynucleotide kinase and γ -³²P ATP (12). After cleavage with endonuclease EcoRI, the labeled fragments were separated by electrophoresis on a 5% polyacrylamide gel. The 300 bp XhoI-EcoRI fragment was extracted and sequenced by the method of Maxam and Gilbert (12). The reproduced portion of the autoradiograph includes the recognition sequence of endonuclease BstPI. Lane M: 5'-labeled XhoI-EcoRI fragment cleaved by BstPI, indicating location of cleavage site. Lane G+M: co-electrophoresis of G-specific cleavage products and marker fragment. The letters G, A, T, C indicate the corresponding sequencing lanes. The lanes M, G+M and G are reproduced twice at two different intensities to facilitate interpretation.

Bst PI (A) - Site :

Bst PI (B) - Site :

Bst PI (C) - Site :

Figure 4. Nucleotide sequences at the endonuclease BstPI sites A, B and C of pQ β HBR DNA. The sequence at site A was known from RNA sequencing studies (15), DNA sequences from the regions of all sites were recently determined (16, and M.A. Billeter, personal communication). These sequences were confirmed by sequence determination using DNA fragments labeled at the 5' ends generated by endonuclease BstPI. Plasmid pQ β HBR was cleaved with endonuclease BstPI and the fragments were treated with bacterial alkaline phosphatase. After terminal labeling with polynucleotide kinase and γ - 32 P ATP, the DNA was further fragmented with endonuclease TaqI. Separation of the products by electrophoresis on a 5% polyacrylamide gel yielded 6 labeled fragments, each representing one 5' end generated by endonuclease BstPI. All 6 labeled fragments were sequenced by the Maxam-Gilbert method (12). The sequences identified in this experiment are indicated by arrows (dotted where the discrimination between C and T residues was doubtful). BstPI sites are indicated by thick lines.

sequence GGTNACC constitutes the recognition site of restriction endonuclease BstPI.

A computer-assisted search through the nucleotide sequences of the DNAs of SV40, of plasmid pBR322 and of the bacteriophages ØX174 and fd, all of which have been determined completely (17-20), revealed that none of these DNAs contains a sequence corresponding to this recognition site (personal communication by J. Shepherd, University of Basel, Switzerland), in agreement with the finding that these DNAs are not cleaved by endonuclease BstPI. As expected, three sites (A, B and C) were detected in a similar search through the complete nucleotide sequence of the genome of bacteriophage Q β (R. Portmann and M. Billeter, personal communication).

d) Determination of the cleavage site.

Since the 5'-terminal nucleotide cannot be deduced unambiguously from a Maxam-Gilbert sequencing gel, the sequences determined from the 5'-labeled BstPI termini do not allow to conclude with certainty which internucleotide bond is cleaved by the restriction enzyme. The exact location of the nucleolytic cleavage can in principle be determined by comparing the electrophoretic mobility of the XhoI-BstPI fragment with the sequence pattern as shown in Fig. 3. Considering that the band indicating the third G residue of the sequence around site B is due to a DNA fragment from which this residue has been removed by chemical degradation (12), it follows that the second of the three Gs must be the 3' end of the labeled XhoI-BstPI fragment, i.e. the cleavage occurs between the two Gs of the palindromic sequence. However, this conclusion is not necessarily valid, since the Maxam-Gilbert fragment and the restriction fragment are not chemically identical; the former carries a 3' phosphate group whereas the latter does not (see below). The influence of a 3' phosphate group on the electrophoretic mobility of a polynucleotide in the denaturing polyacrylamide gel system described by Maxam and Gilbert (12) was not known. Therefore, the site of cleavage was confirmed by determination of the 5' terminal dinucleotide at the ends created by cleavage with endonuclease BstPI.

Plasmid P β G DNA, which contains a single BstPI site, was

cleaved with endonuclease BstPI, treated with phosphatase and labeled at the 5' termini with polynucleotide kinase and γ -³²P ATP. The labeled DNA was digested with DNase I, and portions of the preparation were in addition subjected to a mild treatment with snake venom phosphodiesterase. A collection of small labeled terminal oligonucleotides was obtained, the shortest of which could be separated by electrophoresis on DEAE-paper at pH 3.5 or 1.9. As shown in Fig. 5, the labeled dinucleotide comigrated at pH 3.5 with the dinucleotide dpGpT (added as an unlabeled marker), but not with the dinucleotide dpGpG. The same result was obtained by electrophoresis at pH 1.9 (not shown). This confirms that the cleavage by endonuclease BstPI occurs between the two G residues of the palindrome. It also demonstrates that an additional 3' phosphate residue on a polynucleotide does not change its electrophoretic mobility in a Maxam-Gilbert gel (assuming that the BstPI cleavage produces 5' phosphorylated termini; cf. next paragraph).

e) Ligation of "sticky ends" created by endonuclease BstPI cleavage.

In order to show that endonuclease BstPI creates ends consisting of 3' hydroxyl and 5' phosphomonoester groups which can be covalently linked by DNA ligase, circular plasmid PBG DNA was cleaved by endonuclease BstPI, resulting in the formation of linear (form III) DNA (Fig. 6, slot a, b). On treatment with DNA ligase this linear DNA form disappeared almost completely. Instead, a major band of recircularized DNA was obtained (Fig. 6, slot c) whose electrophoretic mobility in the agarose-ethidium bromide gel was similar, but not identical, to the original circular DNA. The small mobility difference between the two forms is probably due to their unequal linking numbers, resulting in a difference in the amount of bound ethidium bromide. Several minor bands of lower mobility probably represent dimer or higher oligomeric species. If this mixture of ligation products was treated with endonuclease BstPI, a homogeneous band of linear (form III) PBG DNA was again produced (Fig. 6, slot d).

This experiment demonstrates that ends generated by endonuclease BstPI are excellent substrates for DNA ligase. Thus,

Figure 5. Identification of the 5' terminal dinucleotide at the BstPI cleavage site of PBG DNA. Analysis of products resulting from enzymatic digestion of DNA cleaved by BstPI and 5'-terminally labeled with ^{32}P phosphate. Endonuclease BstPI-digested PBG DNA was terminally labeled with γ - ^{32}P ATP and polynucleotide kinase to a specific radioactivity of about 2.5×10^5 dpm/ μg . Samples b and e: 0.15 μg terminally labeled DNA and 5 μg carrier calf thymus DNA were incubated with 1.5 μg DNase I in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 (20 μl) for 2 h at 37°C . 5 μl portions were mixed with 0.25 OD₂₆₀ units of the dinucleotide dpGpT (sample b) or dpGpG (sample e). Samples c and f: 0.15 μg terminally labeled DNA and 20 μg carrier calf thymus DNA were incubated with 3 μg DNase I in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 (15 μl) for 10 min at 37°C . After addition of 0.25 μg snake venom phosphodiesterase (SVP) the incubation was continued at 37°C for 10 min. 3 μl portions were mixed with 0.25 OD₂₆₀ units of the dinucleotide dpGpT (sample c) or dpGpG (sample f). Sample a: dpGpT (0.25 OD₂₆₀ units). Sample d: dpGpG (0.25 OD₂₆₀ units). Samples were spotted on DEAE paper (Whatman DE81) and electrophoresis was performed in pyridine-acetate buffer (pH 3.5, ref. 13) at 2000 V for 6 h. After drying, the marker dinucleotides were detected visually under ultraviolet illumination. The DEAE paper sheets were exposed for autoradiography on Fuji RX X-ray film. The UV-absorbing spots are drawn in as circles. O, origin.

Figure 6. In vitro ligation of "sticky ends" generated by endonuclease BstPI. Endonuclease BstPI-cleaved P β G DNA (0.5 μ g) was incubated in 20 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 10 mM DTT, 0.5 mM ATP (40 μ l) with excess T₄ DNA ligase at 16°C for 3 h. The mixture was extracted with phenol and chloroform and the DNA was precipitated by addition of NaCl (final concentration 0.1 M) and 2 vol ethanol. Half of the sample was cleaved again with endonuclease BstPI. The samples were analyzed by electrophoresis on 1% agarose gels in Tris-acetate buffer (pH 7.8) as described in the Methods section, but gel and buffer contained 0.5 μ g/ml ethidium bromide. a) Original circular P β G DNA (form I). b) P β G DNA cleaved with endonuclease BstPI (form III). c) P β G DNA cleaved with endonuclease BstPI and religated with DNA ligase. d) Cleavage of the religated P β G DNA by endonuclease BstPI.

the nucleolytic cleavage by BstPI occurs on the 3' side of the phosphodiester residue, as reported for all other restriction endonucleases of class II. The DNA sites regenerated by enzymatic ligation are perfectly cleavable by the same restriction enzyme. The results do not allow to decide if imperfectly matching single stranded ends could also be ligated to some extent, nor if heteroduplex sites created by such ligations could be cleaved by endonuclease BstPI.

DISCUSSION

We have purified and characterized from *B. stearothermophilus* a restriction endonuclease (class II) which exhibits novel and interesting properties. Its recognition site: 5'-G-G-T-N-A-C-C-3' covers seven base-pairs and consists of two palindromic elements 3'-C-C-A-N-T-G-G-5' of 3 base pairs each separated by a middle base pair which can be of any type. The enzyme introduces staggered cleavages between the two G residues of the recognition sequence of each strand, resulting in the formation of 5' ends which are single-stranded over a length of 5 nucleotides and carry 5'-phosphate groups. The ends can be ligated with high efficiency by DNA ligase from *T*₄-infected *E. coli*. It seems very likely that perfectly matching ends will be ligated much more efficiently than ends carrying imperfectly complementary sequences. Such a specificity could have useful applications in certain in vitro recombination experiments, e.g. by allowing the selective insertion of a piece of DNA into a vector. As expected, DNA sites resulting from the ligation of endonuclease BstPI generated ends are again cleavable by this enzyme. Of further interest is the fact that several viral and plasmid DNAs commonly used as vectors (e.g. pBR322, SV40, fd) do not contain a BstPI restriction site.

Shortly prior to the submission of this manuscript we became aware of a report that an enzyme with a similar recognition sequence was isolated from *Enterobacter cloacae* (22).

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