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A specific endonuclease from Bacillus caldolyticus

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ABSTRACT

The purification and characterization of a new restriction endonuclease, BclI from the extreme thermophile Bacillus caldolyticus is reported. This enzyme recognizes the sequence

5'-T↓-G-A-T-C-A-3'

3'-A-C-T-A-G↑-T-5'

and cleaves at the positions indicated by the arrows.

INTRODUCTION

Restriction endonucleases are rapidly becoming indispensable in the genetic analysis and manipulation of DNA molecules, and their occurrence is widespread in the bacterial kingdom (1).

Since the discovery of the thermostable enzyme TaqI from the thermophilic bacterium Thermus aquaticus YT1 (2,3) we have screened a variety of thermophilic microorganisms for the presence of restriction endonucleases.

This paper describes the identification and purification of a new restriction endonuclease BclI from the extreme thermophile Bacillus caldolyticus.

MATERIALS AND METHODS

Phosphocellulose P11 and DEAE-cellulose (DE-52) were obtained from Whatman Ltd., Maidstone, England; Sephacryl S-200 from Pharmacia, London, England; agarose from BioRad Ltd., Bromley, England; PM10 membranes from Amicon, High Wycombe, England; ethidium bromide and phenylmethylsulphonyl fluoride (PMSF) from Sigma Ltd., London, England. Pancreatic DNAse, venom phosphodiesterase, and alkaline phosphatase from Worthington. Other chemicals were purchased from BDH Chemicals Ltd., Poole, England.

De-ionized water, autoclaved at 121°C for 40 min to remove traces of

protease (PMSF resistant) and nuclease, was used for all buffers. Phosphocellulose was activated and equilibrated as described by Greene et al. (4) and DE-52 cellulose as recommended by the manufacturer.

Agarose (0.8% w/v) in 90 mM Tris-HCl, 90 mM boric acid, 3 mM Na_2EDTA, pH 8.4, and containing 0.5 µg/ml ethidium bromide was autoclaved for 15 min at 121°C to obtain a homogeneous gel and was used for assaying endonuclease activity throughout the purification.

Adenovirus-2 (Ad2) DNA was a gift from Dr. P. Gallimore, Birmingham University, England, and lambda phage (C1857) was purified according to the method of Bingham et al. (manuscript in preparation).

Protein estimations were carried out according to the method of Lowry (5) and SDS-polyacrylamide gel electrophoresis as described by Laemmli (6).

B. caldolyticus was grown at 65°C with vigorous aeration in the medium described by Sargeant et al. (5).

Endonuclease Assay. BclI activity was assayed throughout the purification by dilution to extinction in 12 mM Tris-HCl, pH 7.4, 12 mM $MgCl_2$, 12 mM NaCl, 0.5 mM dithiothreitol, 2 mg/ml gelatin, 50% glycerol and the smallest quantity of enzyme that would completely digest 0.5 µg Ad2 DNA in 30 min at 50°C was determined (1 unit of endonuclease). Incubations were carried out at 50°C by the addition of 5 µl diluted enzyme to 15 µl 12 mM Tris-HCl, pH 7.4, 12 mM $MgCl_2$, 0.5 mM dithiothreitol, containing 0.5 µg Ad2 DNA. The reaction was terminated after 30 min by the addition of 2 µl 0.2 M Na_2 EDTA, pH 8.0. Unless otherwise stated, electrophoresis was carried out in 0.8% agarose slab gels as described previously (8).

Small-scale extraction of B. caldolyticus were carried out by suspending 10 g cell paste in 30 ml 25 mM Tris-HCl, pH 8.0, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, and the cells broken by sonication (8 x 30 sec) at 20 KH_z, 5A while maintaining the temperature below 15°C. Cell debris was removed by centrifugation at 40,000 x g for 60 min (4°C) and the supernatant adjusted to pH 7.4.

Purification of BclI was effected by suspending 200 g cell paste in 500 ml 25 mM Tris-HCl, pH 8.0, 0.5 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF and passing twice through a Manton-Gaulin homogenizer at 500 Kg/cm^2. The homogenizer was washed with 100 ml of the above buffer to remove the remaining disrupted cells. Cell debris was removed by centrifugation at 25,000 x g for 60 min (4°C), and the supernatant adjusted to pH 7.4.

The extract (550 ml) was loaded onto a 13 l Sephacryl S-200 gel filtration column (90 x 14 cm) equilibrated in 25 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF and eluted with the same buffer at 660 ml/hr. Fractions (330 ml) were collected and those showing endonuclease activity were pooled (2.5 l) and concentrated to 250 ml with an Amicon CH_3 hollow fiber concentrator containing a PM 10 membrane.

The concentrated pool was dialyzed against 40 l of 20 mM potassium phosphate pH 7.4, 0.1 mM Na_2 EDTA, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF (KP buffer) for 17 hours, and loaded onto a 600 ml DE 52 cellulose column (19 x 6.4 cm) equilibrated in the same buffer. The column was then washed with 1 l KP buffer, eluted with a 4 l linear gradient of 0-600 mM NaCl in KP buffer at 250 ml/hr and fractions (110 ml) were collected. Fractions showing endonuclease activity were pooled (1.5 l) and concentrated to 25 ml against a PM 10 membrane as before.

The concentrated DE-cellulose pool was diluted 1:5 by the addition of 1 l KP buffer and 150 g wet wt (4.5 g dry wt) phosphocellulose equilibrated in KP buffer was added. The suspension was stirred for 1 hr and the resin collected in a 9 x 6.5 cm column, and washed with 400 ml KP buffer containing 50 mM NaCl. The endonuclease was eluted with a 2 l linear gradient of 50 mM to 1 M NaCl in KP buffer at 125 ml/hr; 10 ml fractions were collected and those showing enzyme activity were pooled (400 ml) and concentrated to 40 ml against a PM 10 membrane.

The concentrated phosphocellulose pool was loaded onto a 1.8 l Sephacryl S-200 gel-filtration column (55 x 6 cm) equilibrated in 25 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, eluted with the same buffer (100 ml/hr) and 6 ml fractions collected. Fractions containing BclI endonuclease were pooled (180 ml) concentrated against a PM 10 membrane to the smallest volume possible (7 ml) and gelatin added to a final concentration of 0.5 mg/ml. BclI was then dialyzed for 17 hrs against 25 mM Tris-HCl, pH 7.4, 0.2 M NaCl, 10 mM 2-mercaptoethanol, 0.1 mM PMSF, 50% glycerol and stored at -20°C.

Kinasing of 5' ends. Fragments (50 µg) generated from BclI digestion of either Ad2 DNA or lambda dam$^-$ DNA were extracted with phenol and dialyzed against 10 mM Tris-HCl, pH 7.9, 1 mM Na_2 EDTA (TE). After concentration, the DNA was dissolved in 100 ul TE and the 5' ends labeled with ^{32}P by a slight modification (A. Efstradiatis, personal communication) of the procedure of Gingeras et al. (1978) (9). Briefly, the fragments

were treated with alkaline phosphatase (1 ug), an obligatory step, for 1 hr at 37° and then 5 ul 0.15M mM $NaPO_4$, pH 9.5 were added to inhibit further phosphatase digestion. To this mixture was added 5 µl 50 mm $MgCl_2$, 10 ul 50 mM dithiothreitol (DTT), 5 µl polynucleotide kinase (a gift of O. Uhlenbeck) and 0.5 umole γ-^{32}P-ATP (1000 Ci/mmole). After incubation at 37° for 1 hr, the end-labeled DNA was extracted with phenol and desalted on G75 Sephadex run in TE. Counts eluting in the void volume were pooled and precipitated with ethanol. Following incubation in 0.1 N NaOH at 37° for 4 hrs to remove contaminating RNA, the DNA was again desalted. The BclI fragments in the void volume were concentrated and dissolved in 100 µl water.

Fingerprinting. An aliquot of the DNA (5 µl) was dried down and dissolved in a reaction mix (5 µl) containing 10 mM Tris-HCl, pH 7.5, 1 mM DTT, and 0.5 mg/ml pancreatic DNAse. After incubation at 37° for thirty minutes, the DNA was fingerprinted using electrophoresis on cellulose acetate at pH 3.5 in the presence of 7M urea in the first dimension and homochromotography (10) on a thin layer of DEAE-cellulose (1:10) eluting with Homomix VI (11) in the second dimension. The 5' terminal oligonucleotides were detected by autoradiography and eluted from the thin layer plate (12) for redigestion.

Analysis of 5'-terminal dinucleotides. The four standard dinucleotides, pGN, were prepared by phosphorylating the corresponding dinucleoside monophosphates (Collaborative Research) with polynucleotide kinase and γ-^{32}P-ATP as described earlier (9). Oligonucleotides from the fingerprint shown in Fig. 3 were incubated in a reaction mix (20 µl) containing 66 mM glycine-NaOH (pH 9.6), 6.6 mM $MgCl_2$, 3.3 mM dithiothreitol, 5 units exonuclease I (a gift of R. Wu) for 30 min at 37° and the products analyzed by electrophoresis on Whatman 540 paper at pH 3.5. The oligonucleotide pGA in Fig. 3 was unaffected by this treatment and was identical with the product of digestion of all longer oligonucleotides. R_Y refers to the electrophoretic mobility with respect to that of the yellow dye orange G.

Analysis of 5'-terminal mononucleotide. A small aliquot (2 ul) of the terminally labeled fragments which had been digested with pancreatic DNAse (see above) was diluted with an equal volume of snake venom phosphodiesterase (5 mg/ml) in 0.05 M Tris-HCl (pH 8.9), 0.01 M and incubated at 37° for 30 min. The mononucleotides were separated by electrophoresis on Whatman 540 paper at pH 3.5 and detected by autoradiography.

RESULTS AND DISCUSSION

Identification of BclI activity. Examination of an extract of B. caldolyticus (10 g), as part of a screening program for new restriction endonucleases, indicated specific cleavage of both lambda and Ad2 DNAs. A larger scale purification was carried out to obtain the endonuclease, BclI, free of contaminating nucleases.

BclI Purification. The enzyme was purified as described in the Methods and Table 1 provides a summary of the results. B. caldolyticus is difficult to completely disrupt but the treatment described releases >70% of the total BclI endonuclease activity. Ad2 DNA was used for routine assays since lambda phage produced in E. coli K12 W3110 is partially modified against the action of BclI. Nucleic acids were removed by gel filtration on Sephacryl S-200, rather than precipitation with polyethylenimine as previously suggested (8), since substantial loss of activity was observed with the latter technique. Gel filtration provided a $2\frac{1}{2}$-fold purification with good recovery of endonuclease and removed most of the nucleic acids. DEAE-cellulose chromatography removed the remaining nucleic acids and resulted in a $1\frac{1}{2}$-fold purification. BclI eluted between 0.25 M and 0.4 M NaCl with a specific activity of 36,250 units/mg protein (Table 1). Phosphocellulose chromatography gave a 10-fold purification and BclI eluted between 0.45 M and 0.6 M NaCl with a specific activity of 350,000 units/mg (Table 1). Slight amounts of nonspecific nuclease present in the phosphocellulose pool were removed by further Sephacryl S-200 gel filtration. BclI elutes at approximately 950 ml with an increase in specific activity to 1.14×10^6 units/mg. Calibration of this column with aldolase (MW 156,000 daltons), bovine serum albumin (68,000), ovalbumin (45,000),

Table 1: Summary of the Purification of BclI Endonuclease

	Protein	Enzyme	Specific Activity	Recovery
	(mg)	Total Units ($U \times 10^{-6}$)	(U/mg)	(%)
Extract	4,200	41	9,760	100
Sephacryl Pool (A)	1,600	38	23,750	93
DEAE-Cellulose Pool	800	29	36,250	71
Phosphocellulose Pool	40	14	350,000	34
Sephacryl Pool (B)	7	8	1,140,000	19

An extract of B. caldolyticus (200g cell paste) was purified as described in the Methods. Protein and enzyme levels were determined at each stage. One unit of enzyme is defined as the amount that will completely digest 0.5 µg Ad2 DNA in 30 min at 50°C.

chymotrypsinogen A (24,700) and cytochrome C (12,400) gave an estimate of the native molecular weight of BclI as 52,000 daltons. SDS-polyacrylamide gel electrophoresis of the peak BclI fraction of the Sephacryl column before the addition of gelatin revealed one major protein band at 25,000 daltons (fig 1). Thus it would appear that the native enzyme is a dimer of identical 25,000 dalton sub-units.

Temperature Optimum of BclI. The optimum growth temperature of B. caldolyticus is 65°C and production of BclI occurs under these growth conditions. It can be anticipated therefore that BclI should be active at temperatures approaching 65°C. However, as shown in Table 2, its optimum assay temperature is 50°C. The BclI phosphocellulose pool, after concentration and dialysis, showed slight nonspecific nuclease degradation of 0.5 µg Ad2 DNA incubated at 50°C with 100 units of BclI. This exonuclease activity increased three-fold when assays were carried out at 65°C, while at lower temperatures (i.e., 37°C), no exonuclease activity could be detected with over 500 units BclI per 0.5 ug DNA. This would indicate that the temperature optimum of the exonuclease is higher than that of the BclI

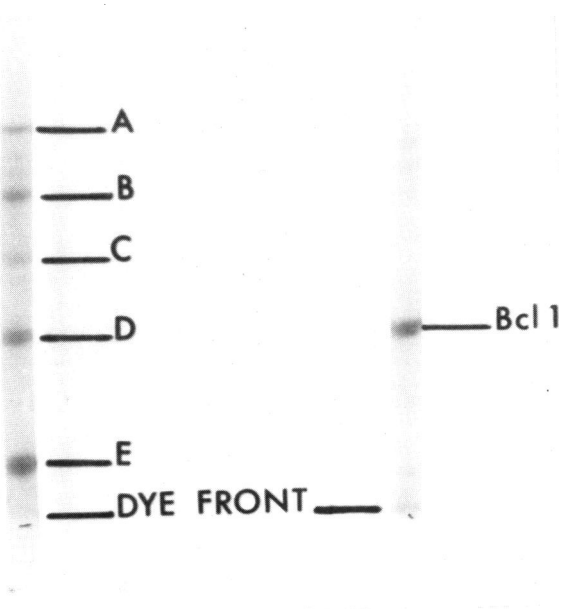

Figure 1: SDS-Polyacrylamide gels (10%) of the peak BclI fraction from the final Sephacryl column, and a set of standards; A: bovine serum albumin (68,000 daltons) B: ovalbumin (45,000) C: deoxyribonuclease (31,000) D: superoxide dismutase (21,000) E: lysozyme (14,400).

endonuclease activity, a property which can be used to advantage, to reduce the possibility of nonspecific degradation.

Characterization. The recognition sequence for BclI was determined by standard procedures, using both Ad2 DNA and bacteriophage lambda (dam^-) DNA. In brief, Ad2 DNA was digested with BclI, the 5'-terminal phosphate removed by treatment with alkaline phosphatase (an obligatory step) and replaced by a ^{32}P phosphate using polynucleotide kinase (Richardson, 1975), and γ-^{32}P-ATP (Glynn and Chappell, 1964). Following digestion of the labeled fragments with pancreatic DNAse, portions were digested with venom phosphodiesterase to identify the 5'-terminal nucleotide (pG), and exonuclease I to analyze the 5'-terminal dinucleotide (pGA) (Table 3). The remainder was fractionated by electrophoresis on cellulose acetate at pH 3.5 in the presence of 7M-urea followed by homochromatography (Figure 2). From this fingerprint, the sequence of the oligonucleotides could be deduced from their mobility shifts and by comparison with oligonucleotides resulting from the analogous experiment performed with MboI fragments (13). The sequence inferred from these data leads to the following recognition sequence for BclI

5'-T↓G-A-T-C-A-3'
3'-A-C-T-A-G↑T-5'

with the sites of cleavage as indicated by the arrows. This places BclI in the same family of restriction endonucleases as BamHI and BglII since all three enzymes have the same internal recognition sequence (GATC). This, in turn, is the recognition sequence for MboI (13). Furthermore, in all four cases, the site of cleavage is identical and tetranucleotide

Table 2: Temperature optimum of BclI endonuclease

Assay Temperature (°C)	Enzyme Activity (Total Units)	Specific Activity (Units/mg)
20	1,000	6,250
37	32,000	200,000
42	50,000	312,500
50	64,000	400,000
55	60,000	375,000
60	25,000	156,250
65	10,000	62,500

BclI activity of the phosphocellulose pool was estimated as described in the methods except incubations were carried out at the above temperatures. One unit is defined as the amount of enzyme that will completely digest 0.5 µg Ad2 DNA in 30 min at the respective temperature.

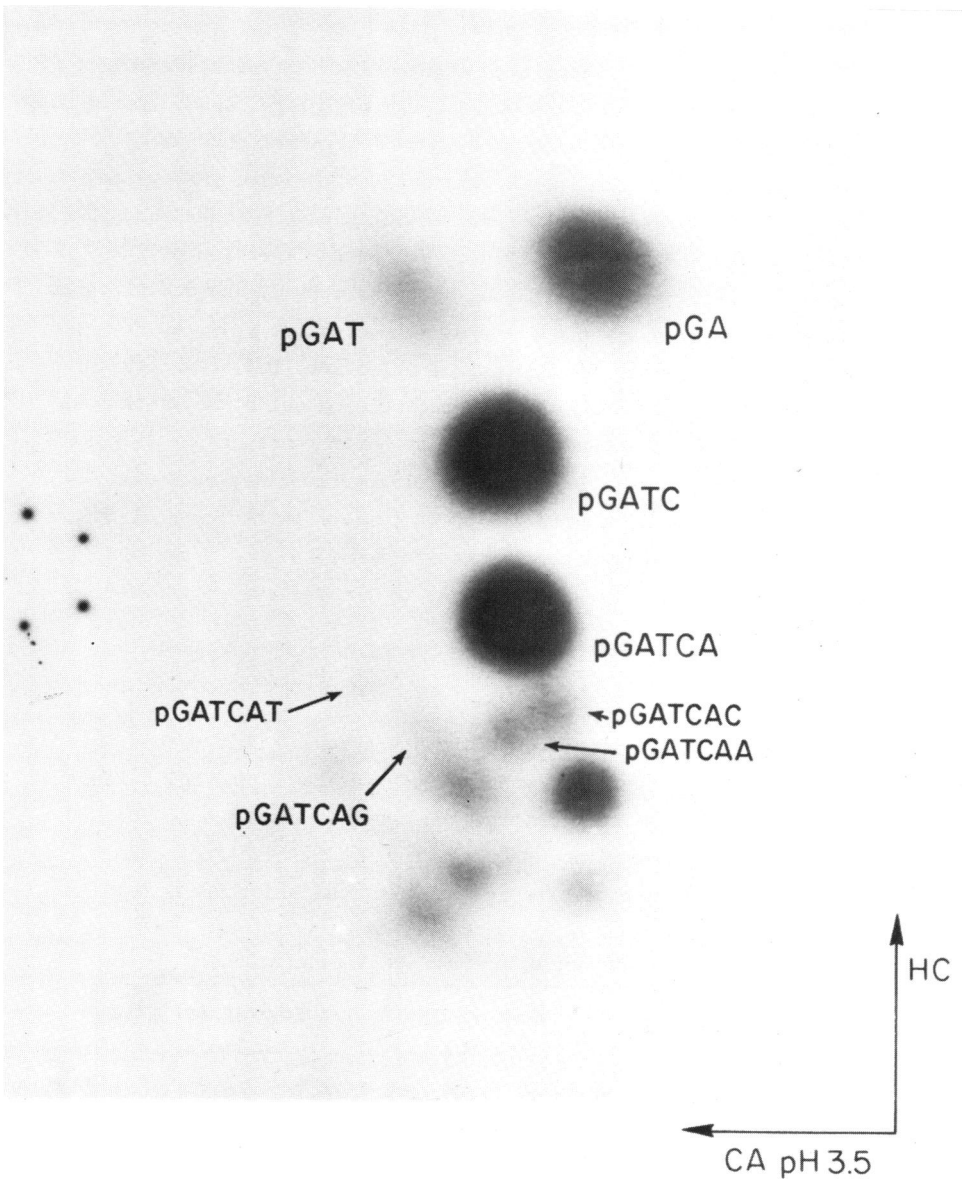

Figure 2: Pancreatic DNAse digestion products of 5'-terminally labeled BclI fragments. Ad2 DNA was digested with BclI and the resulting fragments labeled at their 5' termini using γ-^{32}P-ATP and polynucleotide kinase. These labeled fragments were digested with pancreatic DNAse and fractionated by homochromatography fingerprinting as described in the Methods. The resulting autoradiogram is shown and the identification of the oligonucleotides is described in the text.

Table 3: Identification of the 5' dinucleotide present after cleavage with BclI.

Dinucleotide	R_Y
pGA	0.55
pGC	0.48
pGG	0.70
pGT	0.81
BclI product	0.54

The labeled 5'-dinucleotide present on BclI fragments and the markers were prepared as described in Methods. R_Y refers to electrophoretic mobility with respect to the marker Orange G.

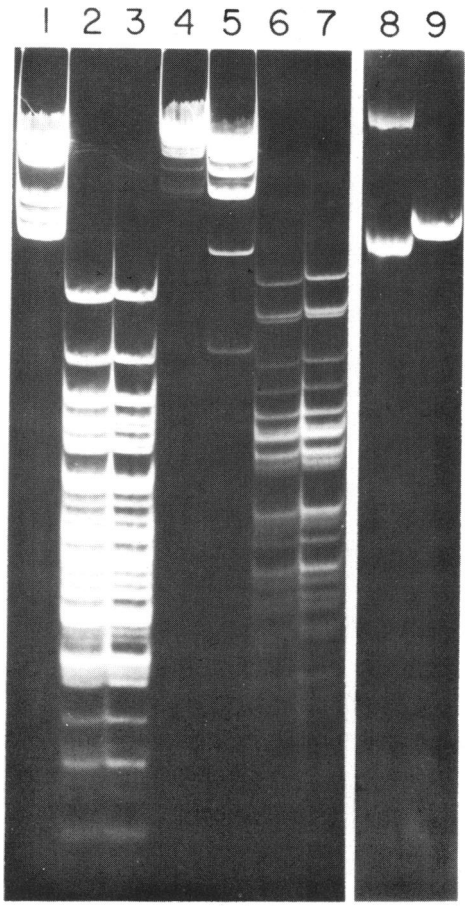

Figure 3: Agarose gel electrophoresis of BclI digests.
(1) BclI on Ad2 DNA; (2) BclI + MboI on Ad2 DNA; (3) MboI on Ad2 DNA; (4) BclI on lambda (wt) DNA; (5) BclI on lambda (dam$^-$) DNA; (6) BclI + MboI on lambda (dam$^-$) DNA; (7) MboI on lambda (dam$^-$) DNA; (8) SV40 DNA; (9) BclI on SV40 DNA.

cohesive termini are generated which should allow the inter-ligation of fragments produced by any of these enzymes.

As shown in Figure 3, BclI cleaves Ad2 DNA at 5 sites and SV40 DNA at a single site (located approximately 235 base pairs away from the BamHI site). ØX174 DNA is not cleaved by BclI (data not shown). Of particular interest is the finding that bacteriophage lambda DNA is resistant to the action of BclI when the phage is grown on a wild type (dam^+) strain of E. coli, but can be cleaved when the phage is grown on a mutant strain (dam^-) which is deficient in its ability to methylate adenosine residues. Such a differential sensitivity has been noted previously for certain other restriction enzymes (14) which recognize the sequence GATC. However, in the cases of BamHI and BglII which also contain this tetranucleotide within their recognition sequences, no such sensitivity occurs.

Other bacterial strains that contain a restriction endonuclease with a specificity similar to BclI include Agrobacterium tumefaciens strain C58 (Sciaky and Roberts, unpublished observations) and Corynebacterium petrophilum (Fisherman, Gingeras, and Roberts, unpublished observations). The precise site of cleavage within the recognition sequence is not known in these cases.

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