An *Sau*3AI restriction endonuclease isoschizomer from *Bacillus cereus*

A.K. Cruz, G. Kidane, M.Q. Pires, L. Rabinovitch*, T.V. Guaycurus* and C.M. Morel+

*Depto. de Bioquímica e Biologica Molecular and *Depto. de Bacteriologia, Instituto Oswaldo Cruz, Av. Brasil 4365, Manguinhos, 21040 Rio de Janeiro, Brazil*

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The isolation and characterization of a restriction endonuclease from *Bacillus cereus* IOC 243 are described. The enzyme recognizes the palindromic sequence 5′-G(met-A,A)TC-3′ as determined by PEI chromatography of pancreatic DNase, snake venom phosphodiesterase digestion products of labelled fragments, analysis of restriction digests from normal and *N*6-methyladenine-free DNA and direct sequence analysis of cloned fragments. The staggered cleavage products with 5′-terminal pGATC extensions are efficiently labelled with polynucleotide kinase and are easily cloned into *Bam*HI sites. The enzyme, denoted *Bce*243, is thus an isoschizomer of *Sau*3AI. Its use and potential advantages in substituting *Sau*3AI are discussed.

Bacillus *Restriction endonuclease* *Molecular cloning* Sau3A *Isoschizomer*

1. INTRODUCTION

Since the discovery of type II restriction endonucleases [1] numerous restriction endonucleases have been purified and characterized [2]. The utility of these enzymes in molecular cloning and structural analysis of DNA continues to be a motive force in the search for new enzymes. We have screened 400 different types of *Bacillus* isolated near Rio de Janeiro, Brazil, for restriction endonucleases. Of these, 6 were found to be good producers of type II endonucleases. We describe here the purification and characterization of one of these from strain IOC 243.

The enzyme recognizes the palindromic sequence 5′-/GATC-3′ and cleaves DNA as indicated by the bar. The protruding 5′-terminus serves as a good substrate for 5′-end labelling in the T4 polynucleotide kinase-catalysed reaction. Furthermore the restriction fragments are easily clonable into *Bam*HI sites.

+ To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Strain culture*

Three 1-l cultures of *B. cereus* IOC 243 were grown in LB medium to late log phase at 37°C. The cells were harvested by centrifugation at 8000 rpm for 10 min. The wet weight of the cells was 12 g.

2.2. *Isolation of restriction endonuclease*

The cells (12 g) were suspended in 2 vols of 10 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol and sonicated at maximum output for 8 min in a model 150 Virsonic cell disrupter using 30-s pulses. The temperature was kept at 4°C throughout the operation. The sonicated cell suspension was then centrifuged for 1 h at 100000 × *g* at 4°C. The supernatant was adjusted to 1 M NaCl by the addition of solid salt. The extract was then applied to a Bio-Gel A-0.5m column with a bed volume of 2.5 × 55 cm and equilibrated with 10 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol and 1 M NaCl. The column was eluted with 5 vols buffer and 10-ml fractions collected

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and assayed for enzyme activity.

Peak fractions were pooled and dialyzed against 10 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol. The dialyzed material was loaded onto a DEAE column (2.5 × 10 cm) equilibrated with 10 mM Tris–HCl (pH 7.5), 10 mM 2-mercaptoethanol. The enzyme was eluted with a 0–0.4 M NaCl linear gradient in the same buffer. Fractions of 5 ml were collected and assayed for enzyme activity. Peak fractions were pooled and subjected to dialysis against 20 mM Tris–HCl (pH 7.5), 7 mM 2-mercaptoethanol and 0.5 mM EDTA. The dialyzed material was loaded onto a heparin–agarose affinity column (1 × 10 cm) [3]. The enzyme was eluted with a gradient buffer of 0–0.8 M NaCl. After enzyme assay, peak fractions were pooled and dialyzed against storage buffer (25 mM Tris (pH 7.5), 200 mM NaCl, 0.2% Triton X-100, 2.5 mM 2-mercaptoethanol, 50% glycerol). It was stored at −20°C after addition of bovine serum albumin to 100 μg per ml.

2.3. *Enzyme assay and characterization of restriction site*

Enzyme activity was assayed by the addition of 3 μl enzyme preparation to 0.3 μg λ DNA in a total reaction volume of 20 μl. The reaction mixture was 10 mM Tris–HCl (pH 7.4), 10 mM $MgSO_4$, 50 mM NaCl, and 1 mM dithiothreitol. Reactions were carried out at 37°C for 30–60 min and stopped by the addition of 3 μl stop buffer (50% glycerol, 5% *N*-lauroylsarcosine, 0.05% xylene cyanol FF and 0.05% bromophenol blue). Digestion products were fractionated in horizontal submarine gels of 0.8–1.5% agarose or in vertical 3–10% polyacrylamide gradient gels. Restriction, end labelling and treatments with pancreatic DNase and snake venom exonuclease were as in [4,5]. Cloning into M13mp8 and sequencing by the chain termination method were performed as in [6].

2.4. *DNA and reagents*

Lambda cI857S7 and plasmid pBR322 DNAs were prepared as in [7,8]. High specific activity [γ-^{32}P]ATP and [α-^{32}P]dATP were obtained from J.C.C. Maia, University of São Paulo, Brazil. Agarose and Bio-Gel A-0.5m and 1.5m were from Biorad. DEAE–cellulose was from Serva. Bacterial alkaline phosphatase, T4 polynucleotide kinase and Klenow's large fragment were from Bethesda Research Laboratories. *N*6-Methyladenine-free λ DNA and T4 DNA ligase were from New England BioLabs.

3. RESULTS

Bacteriophage λ DNA and pBR322 were digested with *Bce*243. The profiles of digestion products after fractionation in agarose or polyacrylamide gels were compared with those generated by several other enzymes. *Bce*243 was found to have the same activity as *Sau*3AI and the enzyme cleaved both normal and *N*6-methyladenine-free DNA, in contrast to the isoschizomer *Mbo*I which cleaves only non-methylated DNAs [9] (fig.1).

To determine precisely the recognition and restriction site of this enzyme, pBR322 DNA was restricted with it and end labelled with polynucleotide kinase and [γ-^{32}P]ATP. One-end labelled

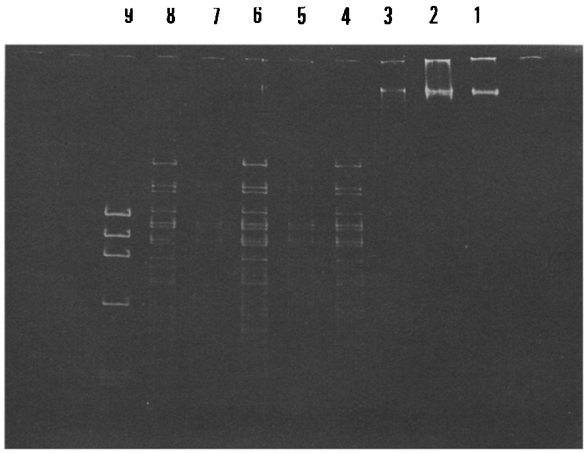

Fig.1. Digestion of normal and *N*6-methyladenine-free bacteriophage λ DNAs with *Bce*243, *Sau*3AI or *Mbo*I. Reaction mixtures were incubated for 1 h at 37°C and analyzed by electrophoresis on 3–10% polyacrylamide gradient gels. The amount of DNA used was 0.9 and 0.5 μg unmethylated and methylated λ, respectively. (Lanes 1,2) Undigested DNA controls; (lanes 3,4) *Kbo*I digestion of normal and unmethylated λ DNA, respectively; (lanes 5,6) *Bce*243 digestion of normal and unmethylated λ DNA; (lanes 7,8) *Sau*3A digestion of normal and unmethylated λ DNA; (lane 9) φX174 (1 μg) digested with *Hae*III as a marker.

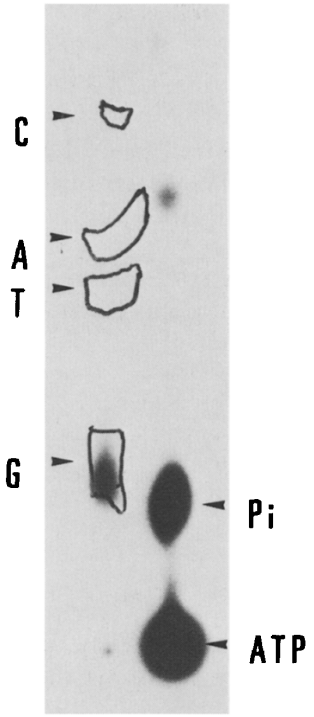

Fig.2. Identification of the 5′-terminal nucleotide of pBR322/*Bce*243/*Rsa*I fragments labelled at the *Bce* end by polynucleotide kinase plus [γ-^{32}P]ATP. The DNA fragment was totally digested with snake venom phosphodiesterase and pancreatic DNase and the nucleotides were chromatographed on a polyethyleneimine thin-layer plate at pH 3.0 [4,5]. Unlabelled 5′-deoxynucleotides were included as internal markers and were detected under UV light. As a reference, a mixture of radioactive P_i and ATP was also analyzed.

molecules generated after a second restriction with *Rsa*I were digested with pancreatic DNase and snake venom phosphodiesterase. The analysis of the digestion products on PEI plates demonstrated that the restriction site has G as its 5′-terminal nucleotide (fig.2).

To ascertain further that the restriction site was GATC, pBR322 DNA was digested with *Bce*243 and fragments in the range of 200–400 bp cloned into the *Bam*HI site of M13mp8. The efficiency of cloning was estimated to be comparable with that of cloning of 200–400 bp long *Sau*3A generated fragments. Furthermore, direct sequence analysis of the cloned molecules at and around the cloning region revealed that the fragments were indeed

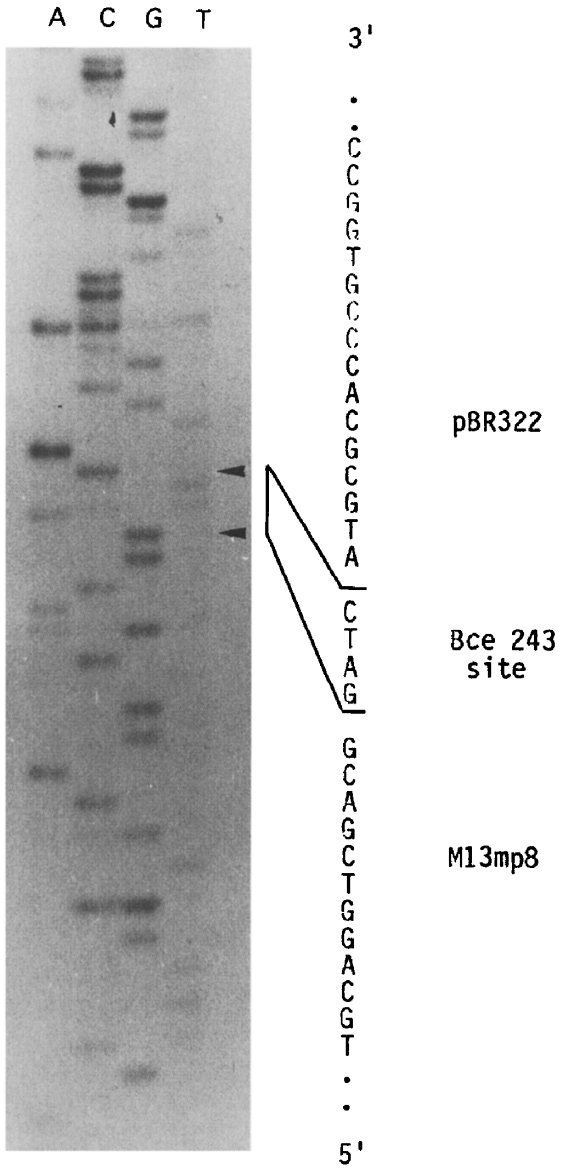

Fig.3. Sequence determination around the *Bam*HI cloning site of a M13mp8-derived recombinant phage containing a pBR322 fragment generated with *Bce*243. Sequencing was done according to the dideoxy chain termination method [6] using an 8% denaturing polyacrylamide gel.

cloned into the *Bam*HI site and that the cloned molecules represented pBR322 sequences cloned in both orientations. Sequence analysis of one of the clones is shown in fig.3.

4. DISCUSSION

We have purified and characterized a *Sau*3A I restriction endonuclease isoschizomer from *B. cereus* IOC 243, isolated near Rio de Janeiro. The enzyme was purified by a modification of standard methods [3,10] and its yield was estimated to be over 1000 units/g cells.

The enzyme was determined to be an isoschizomer of *Sau*3A I by several criteria. Firstly, the restriction profiles of DNAs cut with this enzyme were identical to those generated by *Sau*3A I. Secondly, pancreatic DNase and snake venom phosphodiesterase digestion revealed that restriction fragments of DNAs treated with *Bce*243 have a G nucleotide at their 5′-termini. Thirdly, DNA fragments produced after restriction with this enzyme are clonable into *Bam*HI restricted vectors, this being verified by direct sequence analysis.

*Bce*243 seems to be a good alternative enzyme to *Sau*3A and its known isoschizomers *Fnu*EI, *Bsr*PII, *Mth*I and *Pfa*I [2]. *B. cereus* IOC 243 is easy to grow, the enzyme is easy to purify and its yield is relatively high. Although its thermostability seems not to differ significantly from *Sau*3AI (not shown), its shelf-life seems to be longer. In fact we never detected the appearance of partial digestion products with enzyme preparations kept for at least 10 months at −20°C, a common finding with most of the commercial *Sau*3A preparations we have used. Its ability to cleave efficiently both undermethylated and normal DNAs can render it very useful in molecular cloning and structural analysis studies.

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