Restriction endonucleases from *Bifidobacterium bifidum*

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Received 10 July 1984; revised version received 12 September 1984

Three restriction endonucleases, *Bbi*I, *Bbi*II and *Bbi*III, have been isolated from *Bifidobacterium bifidum*. The recognition and cleavage specificity of *Bbi*II was determined to be 5′-GR↓CGYC-3′, identical to that of *Acy*I isolated from a cyanobacterium *Anabaena cylindrica*. The other two enzymes, *Bbi*I and *Bbi*III, were found to be isoschizomers of *Pst*I and *Xho*I, respectively.

Site-specific endonuclease *Isoschizomer* *DNA sequencing* *Molecular cloning* *5′-GR↓CGYC-3′* *5′-Terminal extension*

1. INTRODUCTION

A large number of restriction endonucleases has been isolated from a variety of bacteria [1], and are widely used in the analysis and in vitro manipulation of DNA molecules. We have screened for restriction endonucleases in the genus *Bifidobacterium* isolated from human and mammals, and found that various strains produce the enzymes with different specificities [2,3].

We describe the purification and characterization of 3 restriction endonucleases, *Bbi*I, *Bbi*II and *Bbi*III, isolated from *B. bifidum*. Among these enzymes, *Bbi*II provides a superior alternative to *Acy*I isolated from the cyanobacterium *Anabaena cylindrica* [4], since *B. bifidum* is much easier to grow than *A. cylindrica*.

2. MATERIALS AND METHODS

DNAs of *Lactobacillus phage Jl, Escherichia coli* phage λ*c*I857*S*am7 and plasmid pBR 322 were prepared as in [2]. φX174 RF DNA, adenovirus 2 (Ad2) DNA and SV40 DNA were purchased from Bethesda Research Laboratories (BRL). Restriction endonucleases were obtained from BRL, New England Biolabs or Takara Shuzo (Kyoto, Japan). Bacterial alkaline phosphatase was from Takara Shuzo, T4 polynucleotide kinase from BRL and nuclease Pl from Yamasa Shoyu (Tokyo, Japan).

2.1. *Strain growth and enzyme purification*

A laboratory strain of *B. bifidum* YIT4007 was grown as in [2]. Yields were 4–6 g/l.

Frozen cells (50 g) were suspended in 4 vols of buffer A (10 mM potassium phosphate (pH 7.4), 7 mM 2-mercaptoethanol, 1 mM EDTA) containing 0.1 mM phenylmethylsulfonyl fluoride and 0.4 M NaCl, and sonicated. Cell debris was removed by centrifugation at 100000 × *g* for 2 h. After treament with 1.2% (w/v) streptomycin sulfate, the supernatant was brought to 70% saturation with ammonium sulfate. The precipitate was collected by centrifugation, dissolved in buffer A and dialyzed against the same buffer.

The dialysate was loaded onto a phosphocellulose (Whatman P11) column (2.5 × 35 cm), and the column eluted with a 1800 ml gradient of 0–0.7 M NaCl in buffer A. *Bbi*I was found in the flow-through. The enzyme activity was recovered by precipitation with ammonium sulfate, dialyzed against buffer B (10 mM Tris-HCl (pH 7.4) 7 mM 2-mercaptoethanol, 1 mM EDTA) and loaded onto

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Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

a DEAE-Sephacel (Pharmacia) column (1.5×25 cm). A 450 ml gradient of 0–0.5 M NaCl was applied to the column. The active fractions eluting at 0.22–0.25 M NaCl were pooled, dialyzed against the same buffer and loaded onto a heparin-Sepharose Cl-6B (Pharmacia) column (1.0×15 cm). A 120 ml gradient of 0–0.7 M NaCl in buffer B was applied to the column. *Bbi*I eluted at 0.12–0.21 M NaCl.

From the phosphocellulose column, *Bbi*II eluted at 0.14–0.19 M NaCl and *Bbi*III eluted as a broad peak at 0.05–0.3 M NaCl. *Bbi*II activity in peak fractions was recovered by precipitation with ammonium sulfate, dialyzed against buffer C (10 mM potassium phosphate (pH 7.0) 7 mM 2-mercaptoethanol, 1 mM EDTA, 0.2 M NaCl) and loaded onto a hydroxyapatite (Biorad) column (1.5×15 cm). The column was eluted with a 270 ml gradient of buffer C with increasing concentration of potassium phosphate up to 0.5 M. *Bbi*II eluted at 0.11–0.17 M potassium phosphate, while *Bbi*III eluted in broad fractions with the peak activity at slightly higher salt concentrations. *Bbi*II was further purified by chromatography on DEAE-Sephacel and heparin-Sepharose CL-6B, in the same way as *Bbi*I. *Bbi*II eluted from a DEAE-Sephacel column (1.0×15 cm) at 0.15 – 0.23 M NaCl when a 0 – 0.5 M NaCl gradient was applied, and eluted from a heparin-Sepharose CL-6B column (1.0×10 cm) at 0.08–0.22 M NaCl when a 0–0.5 M NaCl gradient was applied. Yields of the purified enzyme were approx. 200 units/g cells, where 1 unit of the enzyme was defined as the enzyme activity required to digest 1 μg of pBR322 DNA to completion at 37°C for 1 h, under the conditions used (see section 2.2).

*Bbi*III eluting between 0.05–0.1 M NaCl from the phosphocellulose column was free of contaminating *Bbi*II activity and was pure enough for the identification of the recognition specificity.

All the purified enzymes were concentrated by dialysis against buffer B containing 50% glycerol and stored at −20°C in the presence of bovine serum albumin (500 μg/ml). These enzyme preparations were active after storage for more than a year.

2.2. *Assay of enzyme activity*

The enzyme assay was essentially the same as in [2]. The reaction mixture contained 10 mM Tris–HCl (pH 7.6) 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.5 μg Jl DNA and 2 μl of column fractions in a total volume of 20 μl.

2.3. *Determination of cleavage specificity of* Bbi*II*

DNA sequence analysis [5] was used to identify the cleavage sites of *Bbi*II on φX174 RF DNA. The details of the procedure are given in section 3.2.

For the identification of the 5′-terminal mononucleotide, φX174 RF DNA was cleaved with *Bbi*II, and the products were labeled at the 5′-end with ^{32}P and separated by electrophoresis on a 3.5% polyacrylamide gel. The labeled fragments were digested to completion with an excess of nuclease Pl at 37°C for 1 h, and the digestion products were analyzed, adjacent to 4 standard nucleotides, by thin-layer chromatography on a PEI cellulose plate in 0.5 M lithium formate (pH 3.0) [6].

3. RESULTS AND DISCUSSION

3.1. *Recognition sequence of* Bbi*I,* Bbi*II and* Bbi*III*

The digestion patterns of various DNAs (λ, φX174 RF, pBR322, Ad2 and SV40) with *Bbi*I were similar to those with *Pst*I [7]. The double digest of λ DNA with *Bbi*I and *Pst*I was indistinguishable from the digest with either enzyme alone (not shown). These results indicate that *Bbi*I is an isoschizomer of *Pst*I, recognizing the sequence 5′-CTGCAG-3′. The double digest of Ad2 DNA with *Bbi*III and *Xho*I was identical to the digest with either enzyme alone (not shown). Another isoschizomer of *Xho*I has been isolated from *B. thermophilus* RU326 (unpublished).

Digestion of λ, φX174 RF, pBR322 and Ad2 DNAs with *Bbi*II gave at least 25, 7, 6 and 25 fragments, respectively, on a 1.4% agarose gel, but SV40 DNA was not cleaved by *Bbi*II. Based on these data, together with the sequencing data for sequenced DNAs [9–12], it was deduced that the recognition sequence for *Bbi*II is 5′-GRCGYC-3′, a sequence recognized by *Acy*I isolated from the cyanobacterium *A. cylindrica* [4].

As expected, the double digest of pBR322 DNA with *Bbi*II and *Bbe*I [2], which recognizes the sequence 5′-GGCGCC-3′, a subset of 5′-GRCGYC-3′, was identical to the digest of *Bbi*II alone (not shown).

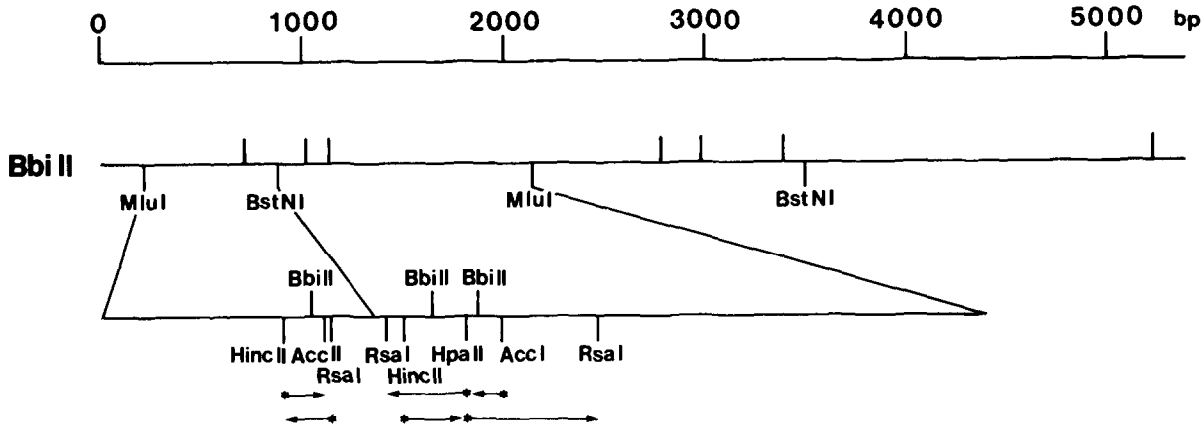

Fig.1. Restriction map of *Bbi*II sites on ϕX174 RF DNA. 3 *Bbi*II sites at coordinate positions at 717 (GGCGTC), 1019 (GGCGCC) and 1133 (GACGCC) were sequenced on both strands. An asterisk* indicates the 5'-terminal label. The arrows represent sequenced regions of the DNA.

3.2. *Cleavage site of* Bbi*II*

The cleavage site of *Bbi*II was determined as follows (see fig.1): ϕX174 RF DNA was digested with a combination of *Bst*NI (an isoschizomer of *Eco*RII) and MluI. Fragments C and D, on which there are 3 *Bbi*II sites at coordinate positions 717 (GGCGTC), 1019 (GGCGCC) and 1133 (GACG CC), were isolated by electrophoresis on a 3.5% polyacrylamide gel. All these sites were subjected to sequence analysis on both strands. To obtain the subfragments containing single *Bbi*II site, fragment C was digested with either *Hpa*II or *Acc*II plus *Hinc*II, and fragment D was with either *Hinc*II or *Rsa*I. The resulting subfragments were isolated by electrophoresis on an 8% polyacrylamide gel, dephosphorylated and ^{32}P-labeled using T4 polynucleotide kinase. Each subfragment was further digested with another restriction endonuclease to yield singly end-labeled fragments, which were then isolated as above.

Fig.2. Determination of the cleavage site of *Bbi*II. A 152 by *Hinc*II-*Hpa*II fragment of ϕX174 RF DNA containing *Bbi*II site at coordinate position 1019 (GGCGCC) (fig.1) was sequenced as in [5]. Since a restriction fragment migrates slightly more slowly than a corresponding, chemically degraded fragment [13], the cleavage site of *Bbi*II lies probably between the R and the C in the sequence of 5'-GRCGYC-3'.

A small portion of a singly end-labeled fragment was cleaved with *Bbi*II, and the cleavage product was subjected to electrophoresis on an 8% polyacrylamide gel, alongside with the products of the same fragment treated by the base-specific cleavage reactions as in [5]. One of the results is given in fig.2. It shows that the cleavage site of *Bbi*II lies probably between the R and the C in the sequence of 5′-GRCGYC-3′.

This was confirmed by the fact that the 5′-terminal mononucleotide was identified as pC after complete digestion with nuclease Pl of the 5′-labeled, *Bbi*II-cleaved ϕX174 RF fragments.

Thus, *Bbi*II has the same recognition and cleavage specificity

5′-GR↓CGYC-3′
3′-CYGC↑RG-5′

as *Acy*I [4].

Since *Bbi*II can be obtained easily and is stable, it may be useful for analysis and cloning of DNA.

ACKNOWLEDGEMENTS

We are grateful to Dr M. Mutai for useful suggestions and encouragement. We thank Dr H. Saito for helpful discussions. We also thank C. Yamaji, T. Watanabe and Y. Shirasawa for their cooperation.

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