Two site-specific endonucleases *Bin*SI and *Bin*SII from *Bifidobacterium infantis*

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Two site-specific endonucleases, *Bin*SI and *Bin*SII, were isolated from *Bifidobacterium infantis* S76e. *Bin*SI was found to be an isoschizomer of *Eco*RII, while *Bin*SII was shown to have the same sequence and cutting specificity as *Bbe*I, 5′-GGCGCC-3′. Both *Bin*SII- and *Bbe*I-generated DNA fragments could be ligated with *Hae*II-generated DNA fragments.

Restriction endonuclease *Isoschizomer* *CC(A/T)GG* *GGCGCC* *3′-Terminal extension* *Ligation*

1. INTRODUCTION

We have screened members of the genus *Bifidobacterium* for the presence of restriction endonucleases, and found that various strains produce these endonucleases with different specificities. In [1] we reported a new restriction endonuclease, *Bbe*I, isolated from a laboratory strain of *Bifidobacterium breve* YIT4006, which recognizes and cleaves the sequence 5′-GGCGCC-3′.

Here, the purification and characterization of two restriction endonucleases, *Bin*SI and *Bin*SII, isolated from *Bifidobacterium infantis* S76e (ATCC15702) will be described.

2. MATERIALS AND METHODS

DNAs of *Lactobacillus* phage J1, *Escherichia coli* phage λ*c*I857*S*am7 and plasmid pBR322 were prepared as in [1]. φX174 RF DNA, adenovirus 2 (Ad2) DNA and SV40 DNA were purchased from Bethesda Research Laboratories. Unmethylated λ DNA was from P-L Biochemicals. Restriction endonuclease *Bbe*I was purified as in [1]. All other restriction endonucleases and T4 DNA ligase were obtained from BRL. Calf intestinal alkaline phosphatase was from Boehringer.

2.1. *Strain growth and enzyme purification*

B. infantis S76e was grown anaerobically at 37°C in modified VL-G medium to early stationary phase [1], harvested by centrifugation and stored at −20°C until use. Yields were 4–6 g/l.

Frozen cells (30 g) were suspended in 4 vol. of buffer A (10 mM potassium phosphate, pH 7.0, 7 mM 2-mercaptoethanol, 1 mM EDTA) containing 0.1 mM phenylmethyl sulfonylfluoride and 0.4 M NaCl, and disrupted by sonication. Cell debris were removed by centrifugation at 100000 × *g* for 2 h. After treatment with 1.2% (w/v) streptomycin sulfate, the supernatant was saturated to 70% with ammonium sulfate. The precipitate was collected by centrifugation at 20000 × *g* for 30 min, dissolved in buffer A and dialyzed against the same buffer.

The dialysate was loaded onto a phosphocellulose (Whatman P11) column (1.5 × 25 cm) equilibrated with buffer A, and the column was eluted with a 320-ml gradient of 0–1.0 M NaCl in buffer A. *Bin*SI eluted both in the flowthrough and at about 0.55 M NaCl as a broad

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peak. The latter fractions, in which the major part of the activity was found, were pooled, dialyzed against buffer B (10 mM Tris–HCl, pH 7.4, 7 mM 2-mercaptoethanol, 1 mM EDTA) and loaded onto a DEAE–cellulose (Whatman DE52) column (1.0 × 10 cm). An 80-ml gradient of 0–0.5 M NaCl in buffer B was applied to the column. *Bin*SI activity eluting at about 0.15 M NaCl was pooled, adjusted to 0.25 M NaCl and loaded onto a heparin–Sepharose CL-6B (Pharmacia) column (1.0 × 5 cm) equilibrated with buffer B containing 0.25 M NaCl. A 40-ml gradient of 0.25–1.0 M NaCl in buffer B was applied to the column. The active fractions eluting at 0.6–0.75 M NaCl were pooled and concentrated by dialysis against buffer B containing 50% glycerol. The enzyme was stored at −20°C after the addition of bovine serum albumin to a concentration of 500 μg/ml.

*Bin*SII eluted from the phosphocellulose column at 0.22–0.27 M NaCl. The enzyme was further purified by successive chromatography on DEAE–cellulose and heparin–Sepharose CL-6B, in the same way as *Bin*SI. *Bin*SII was eluted from a DEAE–cellulose column at 0.19–0.21 M NaCl and from a heparin–Sepharose CL-6B column at 0.38–0.48 M NaCl, when a 0–0.7 M NaCl gradient in buffer B was applied. The purified enzyme was concentrated by dialysis against buffer B containing 50% glycerol and stored at −20°C in the presence of bovine serum albumin (500 μg/ml).

2.2. *Assay of* Bin*SI and* Bin*SII*

The reaction mixture contained 10 mM Tris–HCl, pH 7.6, 10 mM $MgCl_2$, 7 mM 2-mercaptoethanol, 0.5 μg of J1 DNA, and 2 μl of column fractions in a total volume of 20 μl. After incubation for 1 h at 37°C, the reaction was stopped by the addition of 2 μl of 0.2 M EDTA. The digestion products were analyzed by electrophoresis on 1% or 1.4% agarose gels as in [1].

2.3. *Recovery of DNA fragments from agarose gels*

The digestion products of pBR322 DNA with either *Bin*SII or *Bbe*I were separated by electrophoresis on a 1.4% agarose gel. DNA fragments were recovered from the gel as in [2], with the exception that DEAE membrane NA45 (Schleicher and Schuell) was used.

2.4. *Dephosphorylation*

SV40 DNA was linearized by digestion with *Hae*II and dephosphorylated with calf intestinal alkaline phosphatase as recommended by the manufacturer.

3. RESULTS AND DISCUSSION

3.1. *Properties of* Bin*SI and* Bin*SII*

The purified preparations of *Bin*SI and *Bin*SII were judged to be essentially free of non-specific nucleases, since DNA samples digested with 15-fold excess of enzyme gave sharp bands on agarose gels. Both enzymes were stored at −20°C for more than a year without significant loss of activity. *Bin*SI was active over a wide range of pH with an optimum around 7.6. The optimal $MgCl_2$ concentration was between 15 and 20 mM. NaCl was stimulatory and most effective at concentrations of 50 to 60 mM, but it was inhibitory above 100 mM. *Bin*SII was also active over a wide range of pH with an optimum around 7.6. The optimal $MgCl_2$ concentration was about 10 mM, and NaCl was inhibitory even at lower concentrations. In these respects, *Bin*SII closely resembles *Bbe*I [1].

3.2. *Recognition sequence of* Bin*SI and* Bin*SII*

Initial characterization of *Bin*SI was carried out with SV40 DNA. Cleavage sites of *Bin*SI on SV40 DNA were located by digesting with *Bin*SI alone and in combination with *Eco*RI, *Hpa*II, *Taq*I, *Hha*I and *Pst*I, respectively, and by mapping against known restriction sites (not shown). These results, together with the tabularized sequencing data for sequenced DNAs in [3], indicate that the recognition sequence for *Bin*SI is 5′-CC($\frac{A}{T}$)GG-3′, a sequence recognized by *Eco*RII. This was confirmed by double digestion of SV40 DNA with *Bin*SI and *Eco*RII, which gave the same pattern of fragments as that obtained with either enzyme alone (fig.1). Digestion of various DNAs (λ, φX174 RF, pBR322, J1, Ad2 and SV40) with *Bin*SI revealed that DNAs isolated from wild-type strains of *E. coli* were only partially digested even after prolonged incubation with excess enzyme. In contrast, unmethylated λ DNA was digested to completion with *Bin*SI (not shown), indicating that *Bin*SI, like *Eco*RII, is inhibited by *mec* methylation [4]. There is, however, another group of *Eco*RII isoschizomers, which is not inhibited by *mec*

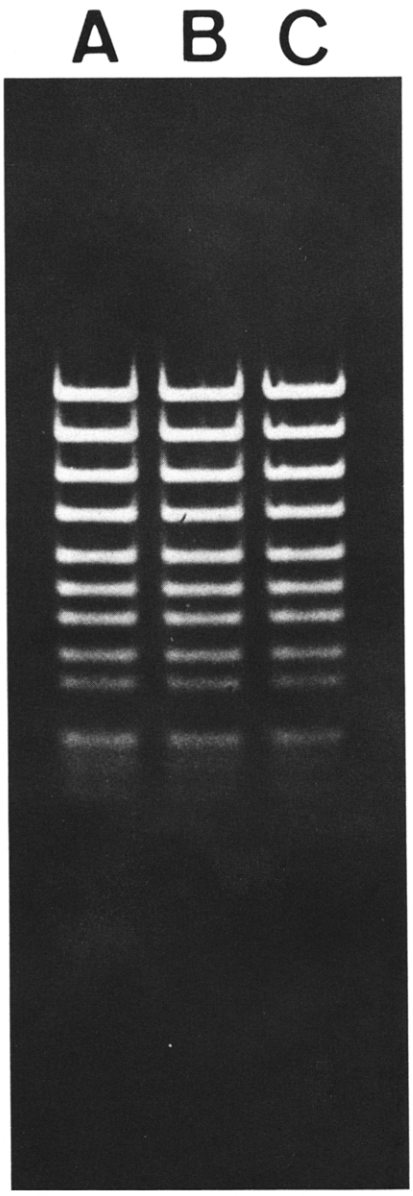

Fig.1. Digestion patterns of SV40 DNA with *Bin*SI and *Eco*RII. SV40 DNA was digested with (A) *Bin*SI; (B) *Bin*SI + *Eco*RII; (C) *Eco*RII. The digests were analyzed on a 1% agarose gel.

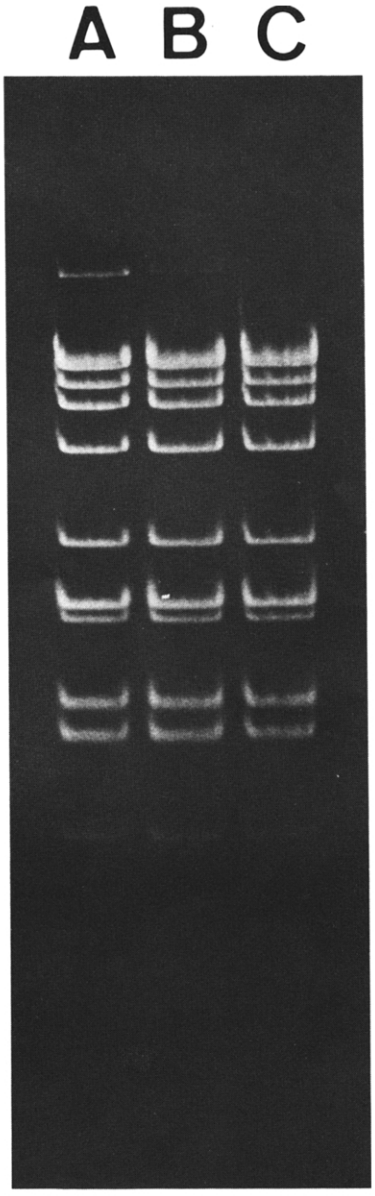

Fig.2. Digestion patterns of Ad2 DNA with *Bin*SII and *Bbe*I. Ad2 DNA was digested with (A) *Bin*SII; (B) *Bin*SII + *Bbe*I; (C) *Bbe*I. The digests were analyzed on a 1% agarose gel.

methylation ([5] and unpublished data cited in [6]).

Digestion patterns of various DNAs with *Bin*SII were identical to those with *Bbe*I. The fragment pattern produced by double digestion of Ad2 DNA with *Bin*SII and *Bbe*I was indistinguishable from that obtained with either enzyme alone (fig.2). These results indicate that *Bin*SII is an isoschizomer of *Bbe*I, which recognizes and cleaves the sequence 5′-GGCGCC-3′, generating 3′-terminal extensions, GCGC-3′ [1].

Restriction endonuclease *Hae*II isolated from *Haemophilus aegyptius* is known to recognize and

cleave the sequence 5′-RGCGCY-3′, generating 3′-terminal extensions, GCGC-3′ [7], identical to those produced by *Bbe*I. In order to examine the cutting specificity of *Bin*SII, the following experiments were made. pBR322 DNA was digested with *Bin*SII, and the largest fragment, 3.6 kb in size, was separated on a 1% agarose gel and recovered from the gel. SV40 DNA, which is about 5.2 kb in size, was linearized by cutting with *Hae*II at its unique site [8], and the ends were subsequently dephosphorylated with calf intestinal alkaline phosphatase. The *Bin*SII-generated, 3.6 kb fragment of pBR322 DNA and *Hae*II-cut, dephosphorylated SV40 DNA were ligated separately or together using T4 DNA ligase at 15°C overnight, and the products were analyzed on a 1% agarose gel. As seen in fig.3, lane A and C, ligation of the 3.6 kb *Bin*SII fragment resulted in dimers, trimers and higher oligomers, while for *Hae*II-cut, dephosphorylated SV40 DNA no ligation products were visible. When these two fragments were mixed and ligated, additional ligation products became visible (fig.3, lane B). One of them corresponds to about 8.8 kb in size expected for a 3.6 kb *Bin*SII fragment and 5.2 kb SV40 DNA ligation product. The same results were obtained when the corresponding 3.6 kb *Bbe*I fragment was used (not shown). These observations demonstrate that both *Bin*SII and *Bbe*II recognize and cleave the sequence 5′-GGCGCC-3′, generating 3′-terminal extensions, GCGC-3′. Therefore, DNA fragments generated by either *Bin*SII or *Bbe*I can be used to form hybrid molecules with *Hae*II-generated DNA fragments.

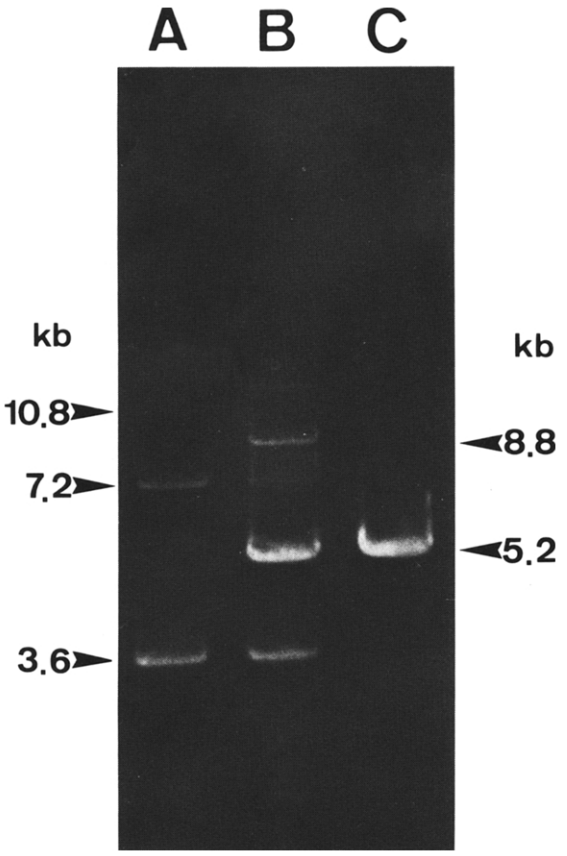

Fig.3. Ligation of *Bin*SII- and *Hae*II-digested DNA. DNA substrates were ligated separately or together, and the products were analyzed on a 1% agarose gel. (A) pBR322 *Bin*SII 3.6 kb fragment. The arrows to the left mark the monomers (3.6 kb), dimers (7.2 kb) and trimers (10.8 kb). (B) pBR322 *Bin*SII 3.6 kb fragment + *Hae*II-linearized, dephosphorylated SV40 DNA (5.2 kb). The arrow to the right-labeled 8.8 kb marks the prominent band of the hybrid ligation product between the pBR 322 fragment and the linearized SV40 DNA. (C) *Hae*II-linearized, dephosphorylated SV40 DNA.

As reported in [1], the isolation of *Bbe*I from *B. breve* YIT4006 involved 4 column chromatographic steps. The major difficulty in the purification of *Bbe*I was the elimination of the trace activity of a second restriction endonuclease, *Bbe*II, and of a DNA topoisomerase, which could not be removed until the last chromatographic step on single-stranded DNA agarose. In comparison, *Bin*SII was easily separated from *Bin*SI and a DNA topoisomerase (T. Khosaka, unpublished) by chromatography on phosphocellulose. Thus, *B. infantis* S76e is a better source of a restriction endonuclease which recognizes and cleaves the sequence 5′-GGCGCC-3′, generating 3′-terminal extensions, GCGC-3′.

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