Volume 10 Number 19 1982 Nucleic Acids Research

New restriction endonucleases from *Acetobacter aceti* and *Bacillus aneurinolyticus*

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Received 24 August 1982; Accepted 8 September 1982

ABSTRACT

Two restriction endonucleases with new sequence specificities have been isolated from Acetobacter aceti IFO 3281 and Bacillus aneurinolyticus IAM 1077 and named AatII and BanII, respectively. Based on analysis of the sequences around the restriction sites, the recognition sequences and cleavage sites of these endonucleases were deduced as below:

AatII : (5') ---N-G -A-C-G-T↓pC-N--- (3')
(3') ---N-Cp↑T-G-C-A- G-N--- (5')

BanII : (5') --N-G -Pu-G-C-Py↓pC-N-- (3')
(3') --N-Cp↑Py-C-G-Pu- G-N-- (5')

INTRODUCTION

A large number of type II restriction endonucleases with different specificities have been isolated from a variety of microorganisms(1). In the course of surveying for new enzymes, we found that Acetobacter aceti IFO 3281 and Bacillus aneurinolyticus IAM 1077 contain two and three kinds of type II enzymes, respectively. Analysis of the restriction patterns yielded by digestion of various DNA substrates and of the sequences around the restriction sites indicated that two of them, named AatII and BanII, had new specificities and the other three were isoschizomers of known enzymes.

MATERIALS AND METHODS

Bacterial strains

Acetobacter aceti IFO 3281 and Bacillus aneurinolyticus IAM 1077 were obtained from Institute for Fermentation, Osaka, Japan and Institute of Applied Microbiology, University of Tokyo, Japan, respectively.

DNAs and enzymes

Phage T4dC and lambda DNAs and plasmids, pAO2, pAO43 and pBR322, were prepared as in the previous paper(2). Phage ϕx174 RF-I and SV40 DNAs were purchased from Bethesda Research Laboratories. Restriction

0305-1048/82/1019-5747$ 2.00/0

endonuclease StuI (3) was a gift from Dr.H.Takahashi of Institute of Applied Microbiology, University of Tokyo. The sources of other enzymes used for restriction analysis and sequence determination have been described previously(2).

Purification procedure of restriction endonucleases from A.aceti and B. aneurinolyticus

A. aceti was grown at 30°C in a medium containing 5 g glucose, 15 ml glycerol, 5 g yeast extract, 5 g bacto-tryptone and 1 g malt extract in one liter(pH 7.2) and B. aneurinolyticus at 30°C in L-broth(2), respectively. The cells were harvested at a late logarithmic phase by centrifugation, washed once with buffered saline and stored at -70°C.

Ten grams of the frozen cells were suspended in 50 ml of 0.05 M tris-HCl(pH 7.5) and 2 mM 2-mercaptoethanol, disrupted by sonication and centrifuged for 60 min at 30,000 rpm. The high-speed supernatant was made 0.1 M NaCl, and then a 10% stock solution of polyethyleneimine, dissolved in water and neutralized, was added to 1%. The precipitate containing mostly nucleic acids was removed by centrifucation, and to the supernatant was added solid ammonium sulfate to 70% saturation. The resulting precipitate was collected by centrifugation, dissolved in 0.01 M potassium phosphate buffer(pH 7.5) and 1 mM 2-mercaptoethanol and dialyzed against the same buffer. The solution was applied on a phospho-cellulose column and chromatographed with a linear gradient of KCl, and type II enzyme activities were assayed as described previously(2). The active fractions were pooled and re-chromatographed on DEAE-cellulose columns(2). Further purification of enzymes was carried out by heparin-agarose column chromatography(4).

Nuleotide sequence determination

Sequence analysis was carried out by the method of Maxam and Gilbert(5). The two dimensional mobility-shift method, which uses electrophoresis and homochromatography, was applied for identification of unique 5'-terminal sequences in a mixture of restriction fragments(2). 5'-terminal nucleotides were analyzed as described earlier(6).

RESULTS AND DISCUSSION

Restriction endonucleases in A.aceti and B.aneurinolyticus

By analysis of fractions resolved by phospho-cellulose column chromatography, we identified two kinds of enzyme activities in A.aceti and three kinds of enzyme activities in B.aneurinolyticus. The enzymes in A.aceti were named AatI and AatII, and those in B.aneurinolyticus BanI,

Table 1 Approximate salt concentrations eluting enzymes from Whatman P-11 and DE-52 and heparin-agarose columns.

Enzymes	P-11 column (KCl,M)	DE-52 column (KCl,M)	Heparin-agarose column (NaCl,M)
AatI	0.35-0.39	0.09-0.11	0.45-0.49
AatII	0.52-0.56	0.21-0.23	-
BanI	0.21-0.25	0.01-0.03	-
BanII	0.42-0.46	0.08-0.10	-
BanIII	0.53-0.57	0.03-0.05	-

BanII and BanIII, respectively. The respective enzyme fractions were purified by DEAE-cellulose column chromatography. AatI was further chromatographed on a heparin-agarose column to remove non-specific nucleases. Approximate salt concentrations that elute these enzymes are given in Table 1.

Viral and plasmid DNAs(pBR322, pAO2, pAO43, ϕx174 RF-I, SV40, lambda and T4dC DNAs) were digested with respective enzymes and the resulting restriction patterns were compared with those of known enzymes. The 5'-terminal sequences of restriction fragments were also analysed. The results of analysis are summarized in Table 2. AatI, BanI and BanIII were found to be isoschizomers of StuI from Streptomyces tubercidicus KCC S-0054(3), HgiCI from Herpatosiphon giganteus Hpg9(cited in ref.1 as an unpublished observation by G.Hobom, H.Mayer and H.Schutte) and ClaI from Caryophanon latum L(7), respectively. The specificities of AatII and BanII were obviously different from those of known enzymes, so that we further analyzed their recognition sequences. Restriction patterns of lambda DNA generated by these two enzymes are shown in Fig.1.

Table 2 Specificities of type-II restriction enzymes from A.aceti and B.aneurinolyticus.

Enzymes	Number of cleavage sites					Specificity
	pBR322	pAO43	ϕx174 RF-I	SV40	lambda	
AatI	0	0	1	7	5	isoschizomer of StuI(3)
AatII	1	0	1	0	7	new enzyme
BanI	9	0	3	1	13	isoschizomer of HgiCI(1)
BanII	2	1	0	2	7	new enzyme
BanIII	1	0	0	0	15	isoschizomer of ClaI(7)

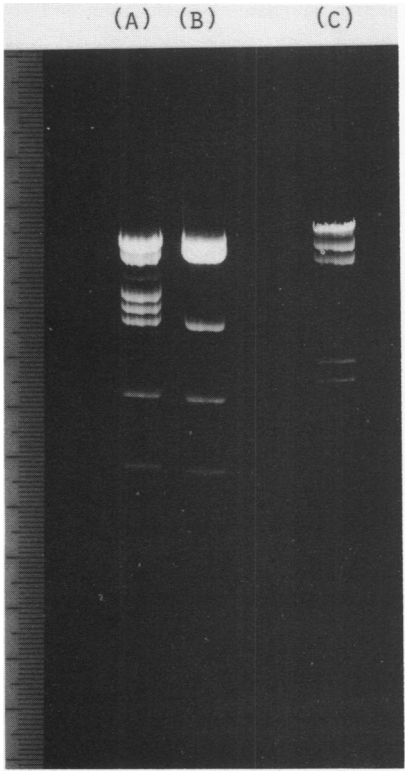

Fig.1 Gel electrophoretic patterns of fragments generated from lambda DNA by digestion with each of AatII (A) and BanII (B). As a reference, the pattern of a HindIII-digest is shown in (C). Lambda DNA was digested with enzymes at 37°C in 0.05 M tris-HCl(pH 7.5), 7 mM $MgCl_2$, 7 mM 2-mercaptoethanol and 0.05 M NaCl. Digests were resolved by electrophoresis on 1% agarose gel in 0.09 M tris-borate(pH 8.3),2.5 mM EDTA and 0.5 ug/ml ethidium bromide.

Recognition sequence of AatII

The recognition sequence of AatII was first analyzed by using pBR322, as this DNA molecule contained a single cutting site. The linearized DNA was labelled at its 5'-termini in polynucleotide kinase reaction and digested with HaeIII. The resulting sub-fragments were isolated and the sequences from the 5'-termini were analyzed by the Maxam and Gilbert method. The sequences obtained were compared with those of pBR322(8) and aligned as in Fig.2. Assuming that AatII is a six-base cutter with two-fold rotational symmetry, the possible recognition sequence is GACGTC. In support of this possibility, this enzyme converts ϕx174 RF-I DNA to a linear form with full length, in which the GACGTC sequence occurs once. By double digestion with other enzymes, it was confirmed that AatII indeed cleaved this DNA at the corresponding site. Thus, we conclude that AatII recognizes the following hexanucleotide sequence and introduces cleavages at the indicated positions.

(5') ---G -A-T-G-T↓pC--- (3')
(3') ---Cp↑A-T-A-C- G--- (5')

Fig.2 Alignment of the 5'-terminal sequences generated by AatII on the pBR322 sequence. The number above the sequences indicate the nucleotide positions from the EcoRI site of pBR322(8). The possible recognition sequence was boxed.

A popular cloning vector pBR322 contains a single AatII cutting site at the upstream region of the bla gene(Nucleotide positions 4,285-4,290 from the EcoRI site of pBR322). This enzyme would be useful for gene cloning by this vector.

Recognition sequence of BanII

Since BanII converts both pBR322 and pAO43 into linear forms, the 5'-terminal sequences of the resulting linear molecules were analyzed and aligned on the sequences of pBR322(8) and pAO43(9,10). As indicated in Fig.3, a common hexanucleotide sequence, GAGCCC, was seen at the cleavage sites. Assuming that BanII is a six-base cutter which recognizes an imperfect palindromic sequence like many other enzymes, the recognition sequence is predicted to be GPuGCPyC. According to the tables of Fuchs et al.(11) in which all possible palindromic sequences of viral and plasmid DNAs

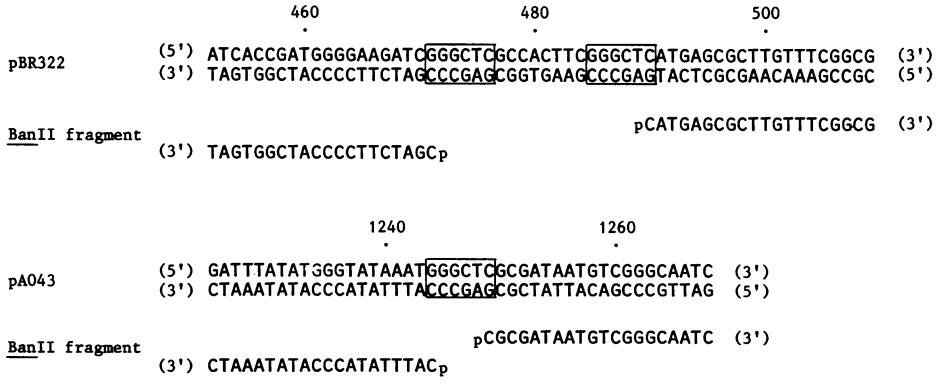

Fig.3 Alignment of the 5'-terminal sequences generated by BanII on the sequences of pBR322 and pAO43 DNAs. The number above the pBR322 sequence indicates the positions from the EcoRI site of pBR322(8), and those above the pAO43 sequence indicates the positions from the left end of Tn903 on pAO43(9,10), respectively. The possible recognition sequence was boxed.

have been listed, such sequences occur two times in SV40 DNA(GAGCCC and GGGCCC) but not in ϕx174 RF-I DNA. In support of this view, BanII cleaves SV40 DNA at two sites but not ϕx174 RF-I DNA. The sizes of fragments generated from SV40 DNA are compatible with those of fragments computed from the sequence data. Thus we conclude that BanII recognizes the following hexanucleotide sequence and introduces cleavages at the indicated positions.

(5') ---G -Pu-G-C-Py↓pC--- (3')
(3') ---Cp↑Py-C-G-Pu- G--- (5')

In the course of preparing this manuscript, we have learned that the enzymes with the same sequence specificity as BanII have been isolated from Bacillus vulgatis OSB816 (BvuI)(12) and Herpetosiphon giganteus HFS101 (HgiJII)(cited in ref.13 as an unpublished observation by N.L.Brown and P.R.Whitehead).

ACKNOWLEDGEMENTS

We thank Dr.K.Soda of this Institute and Dr.T.Tochikura of Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, for providing various bacterial strains. This work was supported by Research Grants from the Ministry of Education, Science, and Culture of Japan.

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