

## Screening of Type II Restriction Endonuclease from Soil Bacteria

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### Summary

Type II restriction endonuclease activity in 405 strains previously isolated as amino acid producing bacteria from soil was screened. The endonuclease activity was found in 37 strains. Five strains were showing relatively high activities [No. 13 (gram positive, chain rod), No. 143 (gram negative, rod), No. 148 (gram positive, chain rod), No. 164 (gram positive, chain rod), and No. 328 (gram positive, rod)] were used for further analysis. To determine the recognition sequences of the type II restriction endonucleases, the enzymes were partially purified with phosphocellulose column chromatography. Digestion patterns of various DNAs (dam<sup>-</sup>λDNA, pBR322 DNA, ϕX174 DNA, ColE1 DNA) with the individual enzymes isolated indicated that they were isoschizomers of *Cla* I (recognition sequence, ATCGAT), *Bci*V I (GTATCC), *Pst* I (CTGCAG), *Ava* II (GGWCC), and *Sau*3A I (GATC).

**Key words:** type II restriction endonuclease, screening, isoschizomer

### Introduction

Restriction endonuclease is an enzyme which recognizes and cuts a double strand DNA at a specific sequence. When a foreign DNA invades a cell (for example infection of virus), restriction endonuclease recognizes that the DNA is different from own DNA and degrades the DNA<sup>1)</sup>. Some bacteria have a self restriction-modification system, and restriction endonuclease is one of the important part of the system.

Restriction endonuclease is classified into three types, based on the manner of its recognition and cleavage of DNA. Type I restriction endonucleases (for example *Eco*B<sup>2)</sup>, *Eco*K<sup>3)</sup>) cleave non-specific sites which are different from recognition sites. Type I restriction endonucleases require a presence of S-adenosylmethionine (SAM), ATP, and Mg<sup>2+</sup> to show their activities<sup>4,5)</sup>. Type II restriction endonucleases (for example *Eco*R I<sup>6)</sup>, *Hind* III<sup>7)</sup>) recognize a double strand DNA at a palindromic sequence and cleave it at specific sites within or adjacent to their recognition sequences. Type II restriction endonucleases require only Mg<sup>2+</sup> to cleave DNAs<sup>8,9)</sup>. Homogeneous DNA fragments containing a target gene can be obtained from long DNA molecules, because type II restriction endonucleases digest DNA at a specific site. Therefore the fragments can be ligated to a vector

(for example plasmid) which is digested by the same enzyme. The cloning vector is introduced into hosts (*Escherichia coli*, Yeasts etc.), and overproduced target products can be obtained<sup>10)</sup>. Thus, type II restriction endonucleases are indispensable tools in the molecular genetics. Type III restriction endonucleases (for example *EcoP* I<sup>11)</sup>, *Hinf* III<sup>12)</sup>) have intermediate properties between type I and type II, and have both endonuclease and methylase activities which require ATP and Mg<sup>2+</sup>. Type III restriction endonucleases cleave at specific sites far away from recognition sites<sup>13,14)</sup>.

More than 3,000 type II restriction endonucleases have been discovered from various microorganisms, however most of them are isoschizomers. More than 200 specific type II restriction endonucleases have been described to date<sup>15)</sup>. Most of restriction endonucleases which recognize 4 to 6 base pairs have been reported. But in considerable 128 kinds of the combination of base sequences, 42 kinds among them have not been reported yet. Thus, if a novel restriction endonuclease which recognizes and cuts unreported combination of base sequences is isolated, it is possible that the enzyme should be newly used as a reagent for gene manipulation.

In this study, to search for novel type II restriction endonuclease or more available isoschizomer which is easy to purify or react, the screening of type II restriction endonuclease was carried out with 405 strains of unidentified soil bacteria which had been used for screening of amino acid producing bacteria in our laboratory. Type II restriction endonuclease activity was detected in 37 strains out of 405 strains, and recognition sequences were determined in 5 strains out of 37 strains. In this paper, we report the characteristics of type II restriction endonucleases produced by these 5 strains.

## Material and Method

### 1. Bacteria and medium

Unidentified soil bacteria were used in this study. Previously they were used for screening of amino acid producing bacteria in our laboratory. They have been isolated from 1994 to 1999.

Bouillon medium (1% meat extract, 1% polypepton, 0.5% NaCl, 0.3% yeast extract, pH 7.0) was used for growth medium. Bacterial strains were stocked on slants of bouillon medium containing 1.7% agar

### 2. Culture conditions

In screening, bacteria were inoculated separately to L tubes containing 10 ml of bouillon medium and incubated at 30°C with shaking for 16 hours.

For partial purification of type II restriction endonucleases, bacteria were grown in 500 ml Erlenmeyer flask containing 100 ml of bouillon medium. They were incubated at 30°C with shaking for 16 hours.

### 3. Preparation of crude extracts

Restriction endonuclease was isolated according to the method of Green *et al*<sup>16)</sup>.

Harvested cells from 10 ml of culture medium were suspended in 500 $\mu$ l of Extract Buffer (EB, 10 mM  $K_2HPO_4$ - $KH_2PO_4$ , 1 mM EDTA $\cdot$ 2Na, 7 mM 2-mercaptoethanol, pH7.0) containing 0.4 M NaCl and sonicated at 4°C with a sonicator [(BRANSON), Duty cycle 10%, Output control 2, 3 minutes]. Cell debris were removed by a microcentrifuge (himac CR15D, HITACHI) at 15,000 r.p.m. for 15 minutes at 4°C. Crude extract was prepared by diluting the supernatant with an equal volume of EB.

#### 4. Partial purification of type II restriction endonucleases

Preparation of phosphocellulose (Whatman P11) was followed the usual method<sup>17)</sup>. Harvested cells from 100 ml of culture were suspended in 25 ml of EB containing 0.4M NaCl and sonicated at 4°C with a sonicator (Duty cycle 30%, Output control 7, 5 minutes). The sonicated cell suspension was centrifuged (55P-72, HITACHI) at 30,000 r.p.m. for 1 hour at 4°C. The supernatant was collected and diluted with EB to the equivalent of 0.1 M NaCl. The crude extract was applied on a phosphocellulose column (1.5 $\times$ 24cm) which had been previously equilibrated with EB containing 0.1 M NaCl. The column was washed with 100 ml of the same buffer, and developed with a linear gradient of 0.1~1.0 M NaCl in 100 ml of EB at a flow rate of 0.3 ml/minute. Four ml fractions were collected.

For desalting, a part of the active fraction (1 ml) was applied on a NAP-5 column (Pharmacia Biotech) equilibrated with EB. The sample was eluted with 2.0 ml of EB, and 500 $\mu$ l fractions were collected.

#### 5. Assay of enzyme activity

Dam<sup>-</sup>  $\lambda$  DNA (NIPPON GENE), isolated from bacteriophage $\lambda$  (cI<sub>875</sub>Sam7), was used as a substrate. Enzyme activity was determined by incubating 12 $\mu$ l of column fractions or the crude extract with 0.25 $\mu$ g of dam<sup>-</sup>  $\lambda$  DNA in a total reaction volume of 50 $\mu$ l at 37°C for 1 hour. Reactions were supplemented with 666 buffer (6 mM Tris-HCl, 6 mM MgCl<sub>2</sub>, 6 mM 2-mercaptoethanol, pH7.5). The reaction was stopped by addition of 3 $\mu$ l of 250 mM EDTA $\cdot$ 2Na.

Agarose gel electrophoresis was followed the usual method<sup>18)</sup> and carried out on 0.7% agarose gels (DOJINDO) using Tris-acetate running buffer (40 mM Tris-acetate, 1 mM EDTA $\cdot$ 2Na, pH8.5) with a submarine type electrophoresis apparatus (ATTO).

#### 6. Digestion of various DNAs

Dam<sup>-</sup>  $\lambda$  DNA, pBR322 DNA (NIPPON GENE),  $\phi$ X174 DNA (NIPPON GENE), and ColE1 DNA (NIPPON GENE) were used as substrates. The condition of digestion of these DNAs was changed depending on reaction time, amounts of substrate, and enzyme.

Double digestion was followed the usual method. Double digestion of dam<sup>-</sup>  $\lambda$  DNA was carried out using the following combinations of the enzyme preparation from indicated strain and commercial enzyme: No.13 and *Cla* I<sup>19)</sup> (TaKaRa), No.143 and *Bci*V I<sup>20)</sup> (New England Biolabs., Inc.), No.148 and *Pst* I<sup>21)</sup> (NIPPON GENE), No.164 and *Ava* II<sup>22)</sup> (NIPPON GENE), and No.328 and *Sau*3A I<sup>23)</sup> (NIPPON GENE).

Table 1. Screening of restriction endonuclease activity from soil bacteria

	Number of strains
Strain used	405
Producing strain	37
Producing Strain No.	(13 <sup>a</sup> , 18, 24, 27, 32 <sup>b</sup> , 38, 43, 44, 51, 60 <sup>b</sup> , 64 <sup>b</sup> , 65, 68, 69 <sup>b</sup> , 72 <sup>b</sup> , 81 <sup>b</sup> , 82, 117, 122, 134, 135, 143 <sup>a</sup> , 148 <sup>a</sup> , 160, 164 <sup>a</sup> , 167 <sup>c</sup> , 170 <sup>c</sup> , 179 <sup>c</sup> , 183, 185, 193 <sup>b</sup> , 196, 197, 205 <sup>c</sup> , 210, 328 <sup>a</sup> , 336)

a : Used strains in this study.

b : Same digestion pattern

c : Same digestion pattern

## Result and discussion

### 1. Screening of type II restriction endonuclease activity

Type II restriction endonuclease activity was screened from 405 strains of unidentified bacteria. Screening of type II restriction endonuclease was carried out whether their crude extract could cleave  $\lambda$  DNA at 37°C for 1 hour. As a result, the enzyme activity was found in 37 strains. These strain No. are shown in Table 1. The digested patterns with 7 enzymes produced by No. 32, 60, 64, 69, 72, 81, and 193 were identical with each other, and No. 81 had the strongest activity. The digested patterns with 4 enzymes produced by No. 167, 170, 179, and 205 were also identical with each other, but it was different from that described above, and No. 170 had the strongest activity. Twenty-eight different types of digested patterns were detected. The enzyme activities of 5 enzymes produced by No. 13, 143, 148, 164, and 328 were higher than others.

### 2. Microbial characterization of type II restriction endonuclease producing bacteria

Microbial characteristics of 5 strains were investigated. Morphology of the 5 strains were observed by microscope, and gram stain, growth in aerobic or anaerobic state, spore formation, motility, and catalase activity were investigated. The genus of these bacteria were estimated based on "Bergey's Manual of Systematic Bacteriology"<sup>24)</sup>. From the

Table 2. Microbial characteristics of restriction endonuclease producing bacteria

Strain No.	13	143	148	164	328
Form	chain rod	rod	chain rod	chain rod	rod
Length ( $\mu\text{m}$ )	4×2	3×1	5×1	4×2	4×2
Aerobe or anaerobe	facultative aerobe	facultative aerobe	facultative aerobe	facultative aerobe	facultative aerobe
Gram stain	+	-	+	+	+
Spore formation	+	-	+	+	+
Motility	-	+	+	-	+
Catalase	+	+	-	+	+
Estimated genus	<i>Bacillus</i>	<i>Yersinia</i>	<i>Sporolacto-bacillus</i>	<i>Bacillus</i>	<i>Bacillus</i>

results shown in Table 2, No. 13, 164, and 328 were estimated to belong to genus *Bacillus*. No. 143 was estimated to belong to genus *Yersinia*. No. 143 was thought to be *Yersinia enterocolitica*. No. 148 was estimated to belong to genus *Sporolactobacillus*, because this bacterium had been isolated from bird feces.

### 3. Partial purification of 5 restriction endonucleases

Partial purification of 5 restriction endonucleases was carried out with phosphocellulose column chromatography. The crude extracts were prepared from 100 ml of culture and applied on the phosphocellulose column. To confirm the enzyme activities, every fractions was incubated with  $dam^- \lambda$  DNA at 37°C for 1 hour. The active fractions of No. 13, 143, and 148 were eluted at about 0.2 M NaCl. The active fractions of No. 164 and 328 were eluted at about 0.4 M NaCl and 0.5 M NaCl, respectively. The active fractions were collected and applied on a NAP-5 column for desalting. Active fraction eluted by a NAP-5 column was used for the determination of recognition sequence in further experiments.

### 4. Determination of recognition sequences

Various DNAs of  $dam^- \lambda$ , pBR322,  $\phi$ X174, and ColE1 were digested with the partially purified enzyme, and number of cleavage sites were determined. The result is shown in Table 3. Number of cleavage sites and the length of DNA fragments were indicated that No. 13, 143, 148, 164, and 328 were isoschizomer of *Cla* I, *Bci*V I, *Pst* I, *Ava* II, and *Sau*3A I, respectively, and recognition sequences of them were 5'-ATCGAT-3', 5'-GTATCC-3', 5'-CTGCAG-3', 5'-GGWCC-3' (W=A or T), and 5'-GATC-3', respectively (Table 4). The consequence was confirmed by double digestion. Double digestion of  $dam^- \lambda$  DNA was carried out using the following combinations of the partially purified enzyme from indicated strain and commercial enzyme: No. 13 and *Cla* I, No. 143 and *Bci*V I, No. 148

Table 3. Number of cleavage sites of various DNAs

Strain No.	Number of cleavage sites			
	$dam^- \lambda$	pBR322	$\phi$ X174	ColE1
13	15	1	0	1
143	26	2	4	4
148	28	1	1	3
164	35	8	1	4
328	116	22	0	19

Table 4. Isoschizomer and recognition sequence

Strain No.	Recognition sequence	Isoschizomer
13	3'-ATCGAT-5'	<i>Cla</i> I
143	3'-GTATCC-5'	<i>Bci</i> V I
148	3'-CTGCAG-5'	<i>Pst</i> I
164	3'-GGW <sup>a</sup> CC-5'	<i>Ava</i> II
328	3'-GATC-5'	<i>Sau</i> 3A I

a : A or T

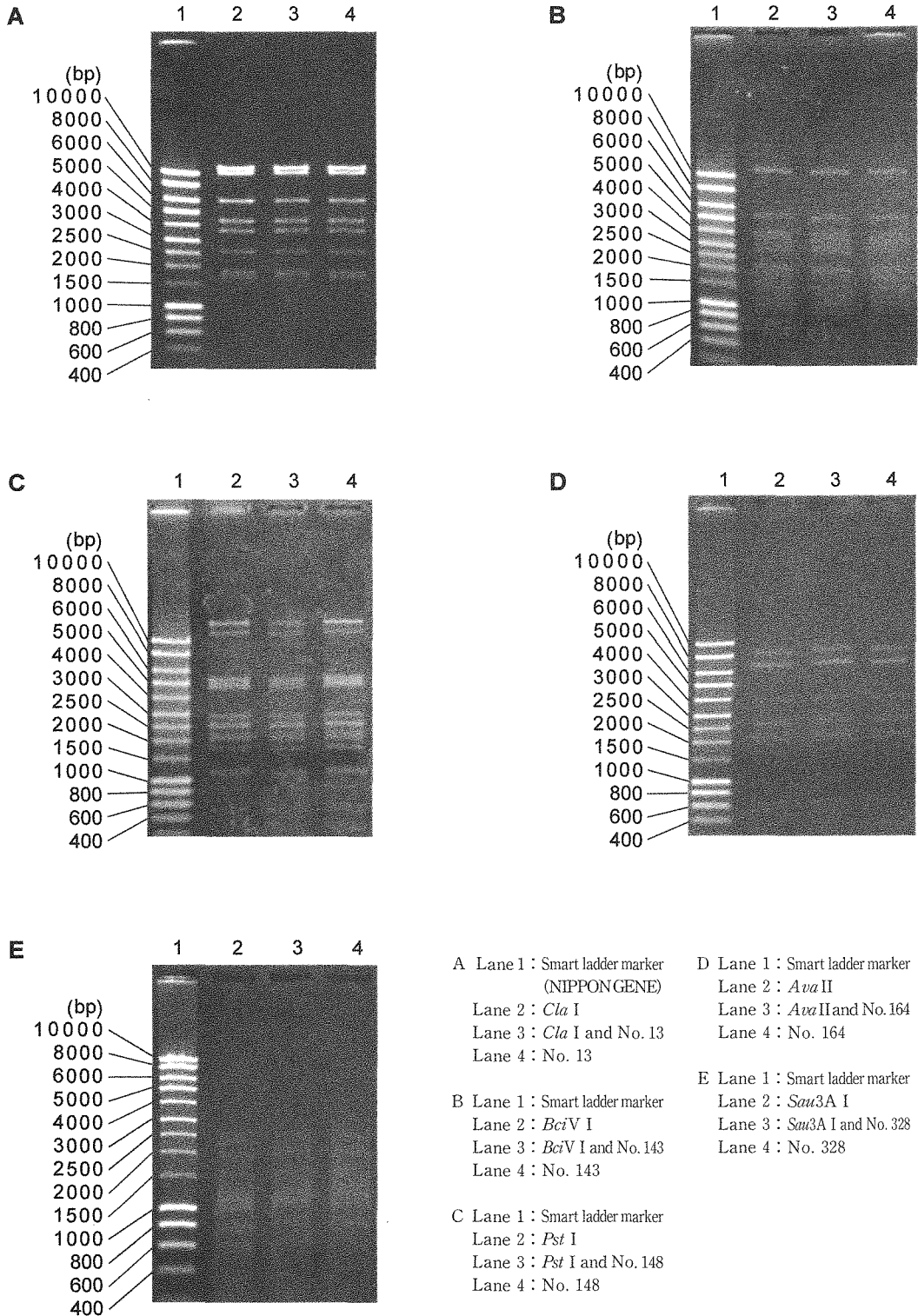


Fig. 1. Single and double digestion agarose gel electrophoresis patterns of *dam*<sup>-</sup>λDNA

and *Pst* I, No. 164 and *Ava* II, and No. 328 and *Sau*3A I. The results are shown in Fig. 1. Each restriction pattern on  $\text{dam}^- \lambda$  DNA matched with the pattern generated by their isoschizomer.

There is no report on the isoschizomer of *Bci*V I. *Bci*V I is produced by *Bacillus circulans*, No. 143 was, however, estimated to be *Yersinia enterocolitica*. Therefore the type II restriction endonuclease produced by No. 143 may be the first reported isoschizomer of *Bci*V I.

No. 148 was estimated to be genus *Sporolactobacillus*, and it produced the isoschizomer of *Pst* I. Many isoschizomers of *Pst* I were isolated from various microorganisms, but there is no report on the type II restriction endonuclease produced by *Sporolactobacillus*. Therefore the type II restriction endonuclease isolated from No. 148 is probably the first enzyme produced by *Sporolactobacillus*. It is interesting to compare the properties of this enzyme with the other isoschizomers of *Pst* I, when the properties of this enzyme have been investigated.

Type II restriction endonucleases isolated from the remaining 23 strains may be investigated as it remains the possibility to find a new enzyme.

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## 土壌細菌由来の II 型制限酵素の検索

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### 摘 要

新規の II 型制限酵素や, 既存のものであってもその精製や反応条件の簡単なものを得ることを目的とし, II 型制限酵素の検索を行った. 検索の対象には, 当研究室で学生実験の際にアミノ酸生産菌の分離に用いた未同定の菌株, 405株を用い, そのうち37株について II 型制限酵素活性が認められた. このうち, No.13 (グラム陽性, 連鎖状桿菌), No.143 (グラム陰性, 桿菌), No.148 (グラム陽性, 連鎖状桿菌), No.164 (グラム陽性, 連鎖状桿菌), No.328 (グラム陽性, 桿菌) の 5 株が生産する II 型制限酵素については, 部分精製を行い, 様々な基質 DNA (dam<sup>-</sup>λDNA, pBR322 DNA, φX174 DNA, ColE1 DNA) と反応させることによりその認識部位の決定を行った. その結果, これらは, それぞれ, *Cla* I (ATCGAT), *Bci* V I (GTATCC), *Pst* I (CTGCAG), *Ava* II (GGWCC), *Sau*3A I (GATC) のアイソジゾマーであることが明らかとなった.