Bull. Inst. Chem. Res., Kyoto Univ., Vol. 60, No. 5~6, 1982

Screening of Type II Restriction Endonucleases

I. Isolation and Characterization of Enzymes from Fifteen Bacterial Strains

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Received September 7, 1982

One hundred and forty-seven bacterial strains were surveyed for the presence of type II restriction endonucleases, and eighteen species of enzymes were successfully isolated from fifteen strains in which enzyme activities were identified. Based on analysis of the restriction patterns generated from viral and plasmid DNAs and of the sequences around the cleavage-sites, four of enzymes named *Aat*II, *Ban*II, *Fok*I and *Mlu*I were found to have new specificities. The remaining fourteen enzymes, named *Aat*I, *Atu*IAMI, *Ban*I, *Ban*III, *Bpr*I, *Eco*ICRI, *Ggl*I, *Gin*I, *Mau*I, *Pai*I, *Pan*I, *Pfl*I, *Ppu*I, and *Spa*I, were isoschizomers of known enzymes.

KEY WORDS: Restriction endonuclease/ Recognition sequence/ Isoschizomer

I. INTRODUCTION

Type II restriction endonucleases are a class of enzymes which introduce double-stranded cleavages at unique sites of DNA by recognizing specific sequences. This type of enzymes have only been identified in bacterial strains and their specificities are known to be strain-specific. Since the collection of different enzymes is of course advantagenous for gene cloning and DNA dissection, many bacterial strains have extensively been surveyed during the last several years, and more than seventy species of enzymes with different specifities have now been isolated.[1,2] The recognition sequences of these enzymes are limited to tetra- to hexa-nucleotides, and most of them have two-fold rotational symmetry. Assuming that enzymes corresponding to all possible sequences of tetra- to hexa-nucleotides with such palindromic structures are present in microorganisms, the number of enzymes are expected to be more than one hundred fifty. Based on this assumption, we carried out screening of new enzymes on bacterial strains which have not been examined yet. As a consequence, we identified enzyme activities in fifteen strains out of one hundred forty-seven strains examined. Enzymes were purified from respective strains and their cleavage-site specificities were analyzed.

II. MATERIALS AND METHODS

Bacterial strains and culture conditions

The sources and strain names used for screening are listed in *Table I. Acetobacter*

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and *Gluconobacter* strains were obtained from Dr. T. Tochikura of Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, some of *Pseudomonas* and *Streptomyces* strains from Toyoboseki Co., and all other strains from Dr. K. Soda of this Institute, respectively. The compositions of culture media were as follows:

a) *Acetobacter* and *Gluconobacter* strains; 0.5% glucose, 1.5% glycerol, 0.5% yeast extract, 0.5% bacto-tryptone, and 0.1% malt-extract (pH 7.2).

b) *Lactobacillus* and *Streptococcus* strains; 0.85% yeast extract, 1.1% glucose, 0.2% KH_2PO_4, 10% tomato juice, 0.1% Tween 80 and 2% $CaCO_3$ (pH 7.2).

c) *Micrococcus, Mycobacterium, Pseudomonas* and *Serratia* strains; 1% meat extract, 1% bacto-tryptone, 2% glucose and 5% NaCl (pH 7.0).

d) *Streptomyces* strains; 2% nutrient broth (Difco), 0.2% yeast extract, 0.8% NaCl, and 0.5% glucose (pH 7.2).

e) All other strains; 1% bacto-tryptone, 0.5% yeast extract, 0.5% NaCl and 0.1% glucose (pH 7.2).

Strains were grown at either 30°C (a to e) or 37°C (c and e) to a late logarithmic phase by vigorous shaking in a NBC-rotary shaker. The cells were then harvested by centrifugation, washed once with buffered saline and stored at −70°C.

Viral and plasmid DNAs and other enzymes

Phage T4dC and lambda DNAs and plasmids, pAO2, pAO43 and pBR322, were prepared as in the previous paper.[3,4] Phage ϕX174 RF-I and SV40 DNAs were purchased from Bethesda Research Laboratories. The sources of other enzymes used for restriction and sequence analyses have been described previously.[5]

Purification of enzymes

Purification of enzymes was carried out by the procedures essentially identical to those previously used.[3~5] 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 in a Tomy Ultrasonic Disruptor and centrifuged for 60 min at 30,000 rpm in a Spinco 50Ti rotor. 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 centrifugation, 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 (0H 7.5) and 1 mM 2-mercaptoethanol, and dialyzed against the same buffer. The solution was applied on a phospho-cellulose column (Whatman, 1 cm×20 cm) and chromatographed with a linear gradient of KCl, and type II-enzyme activities were assayed. The active fractions were applied on a DE52-cellulose column (Whatman, 1 cm×15 cm) and chromatographed with a linear gradient of KCl. Further purification of enzymes was carried out by heparin-agarose column chromatography.[6]

Assay of enzyme activities

One to five μl of fractions were added to 30 μl reaction mixtures containing 0.01 M tris-HCl (pH 7.5), 7 mM $MgCl_2$, and 1 μg of viral or plasmid DNAs. After

incubation for 30 min to 2 hrs at 37°C, reaction was terminated by adding 5 μl of 0.05 M EDTA, 15% Ficoll and 0.02% bromophenol blue. The solutions were electrophoresed for 2 to 4 hrs at 10 V/cm on horizontal slab gels of 1% agarose, dissolved in 90 mM tris-borate buffer (pH 8.3), 2.5 mM EDTA and 0.5 μg/ml ethidium bromide.

Nucleotide sequence determination

Sequence analysis was carried out by the method of Maxam and Gilbert.[7] 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.[8] The 5'-terminal nucleotides were analyzed as described earlier.[3]

III. RESULTS AND DISCUSSION

For the primary screening of enzymes, about 1 g of frozen cells were suspended in 10 ml of tris-HCl (pH 7.5)81 mM 2-mercaptoethanol, disrupted by a sonicator, and centrifuged for 60 min at 30,000 rpm. The cleared supernatant was directly applied onto a small phospho-cellulose column (0.5 cm × 1 cm), equilibrated with potassium phosphate buffer (pH 7.5)-1 mM 2-mercaptoethanol-5% glycerol (buffer A). After washing the column with 5 ml of buffer A, adsorbed proteins were eluted with 2 ml of 1 M KCl in buffer A. The flow-through fraction and 1 M KC:-eluate were submitted for assay of type II-enzyme activities. All the strains listed in *Table I* were examined by this way, and enzyme activities were detected in fifteen strains.

As the next step, the extracts from the respective strains, in which some enzyme activities were detected, were fractionated by phospho-cellulose column chromatography and enzyme activities in fractions were assayed. The activities in some strains were resolved into two or three different enzymes. The active fractions which generate unique restriction patterns were pooled, and purified by DEAE-cellulose and heparin-agarose columns chromatography. The respective enzymes were con-, centrated by dialysis against buffer A containing 50% glycerol, and named according to the nomenclature by Smith and Nathans.[9]

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 I*. Among the eighteen enzymes isolated, *Aat*II from *Acetobacter aceti* IFO 12536, *Ban*II from *Bacillus aneurinolyticus* IAM1077, *Fok*I from *Flavobacterium okeanokoites* IFO12536 and *Mlu*I from *Micrococcus luteus* IFO12992 were found to have new specificities. The detailed data for the specificities of these enzymes have been published elsewhere.[4,10] The remaining fourteen enzymes were isoschizomers of known enzymes.

We could identify type II enzyme activities only in fifteen strains out of one hundred forty-seven strains examined, but this does not mean that other strains are lacking in this type of enzymes. In our screening procedure, we first fractionated extracts into the adsorbed and non-adsorbed fractions by phospho-cellulose. In

Table I. Bacterial strains assayed for the presence of type II restriction endonucleases

Bacteria	Sources	Enzymes	Specificity
Acetobacter aceti	IFO 3281	*Aat* I AGG↓CCT	isoschizomer of *Stu* I (11)
		Aat II GACG↓T	new enzyme (10)
Acetobacter ascendens	IFO 3188	—	
Acetobacter kuetzingianus	IFO 3222	—	
Acetobacter melanogenes	IFO 3293	—	
Acetobacter tubidans	IFO 3225	—	
Achromobacter georgioplitanum	ATCC 23203	—	
Achromobacter obae	ICR 0870	—	
Achromobacter polymorph	ICR 0880	—	
Achromobacter superficialis	ICR 0890	—	
Acinetobacter species	TOYOBO	—	
Aerobacter aerogenes	AKU 028	—	
Aerobacter aerogenes	IFO 3220	—	
Agrobacterium randiobacter	IAM 1526	—	
Agrobacterium tumefaciens	IAM B-26-1	*Atu* IAMI ?	
Alcaligenes brookeri	IFO 12948	—	
Alcaligenes faecalis	IAM B-141	—	
Alcaligenes faecalis	IFO 3160	—	
Alcaligenes viscolactis	IAM 1517	—	
Arthrobacter atrocyaneus	IFO 12670	—	
Arthrobacter ureafaciens	IAM 625	—	
Bacillus amyloliquefaciens K-50	TOYOBO	—	
Bacillus aneurinolyticus	IAM 1077	*Ban* I C↓GPyPuCC	isoschizomer of *Hgi* CI (1)
		Ban II GPuGCPy↓C	new enzyme (10)
		Ban III AT↓CGAT	isoschizomer of *Cla* I (12)
Bacillus circulans	ICR 1560	—	
Bacillus licheniformus	IFO 12200	—	
Bacillus licheniformus	IAM 11054	—	
Bacillus mesentericus var. *flavus*	IFO 3028	—	
Bacillus reseus Migula	IFO 3041	—	
Bacterium mycoides	IFO 3040	—	
Brevibacterium ammoniagenes	IFO 12071	—	
Brevibacterium ammoniagenes	IFO 12072	—	
Brevibacterium divaricatum	NRRL 2311	—	
Brevibacterium linens	IFO 12141	—	
Brevibacterium linens	IFO 12147	—	
Brevibacterium protophormiae	IFO 12128	*Bpr* I ?	
Cellulomonas flavigena	IFO 3747	—	
Cellulomonas flavigena	IFO 3754	—	
Cellulomonas flavigena	IFO 12680	—	
Corynebacterium michiganse	ICR 2211	—	
Corynebacterium pseudodiphitheriticus	ICR 2210	—	

Corynebacterium sepedonicus	ICR	2200	—		
Corynebacterium xerosis	IFO	12684	—		
Erwinia aroideae	IFO	3830	—		
Erwinia carotovora	IFO	3057	—		
Escherichia coli crookes	ICR	0010	—		
Escherichia coli E8	ICR	0030	—		
Escherichia coli E9	ICR	0040	—		
Escherichia coli 2bT	ICR	0020	*Eco* ICRI	GAGCTC	isoschizomer of *Sst* I (13)
Escherichia coli freudii S-96	ICR	0070	—		
Flavobacterium arborescens	IAM	1100	—		
Flavobacterium lutescens	IFO	3084	—		
Flavobacterium lutescens	IFO	3085	—		
Flavobacterium marginata	IFO	3066	—		
Flavobacterium okeanokoites	IFO	12536	*Fok* I	GGATG	new enzyme (4)
Flavobacterium suavealens	IFO	3752	—		
Gluconobacter gluconicus	IFO	3285	*Ggl* I	?	
Gluconobacter industricus	IFO	3260	*Gin* I	GGATCC	isoschizomer of *Bam* HI (14, 15)
Gluconobacter liquefaciens	IFO	12388	—		
Gluconobacter suboxydans	IFO	3130	—		
Gluconobacter suboxydans	IFO	3172	—		
Gluconobacter suboxydans var. α	IFO	3254	—		
Lactobacillus acidophilus	IFO	3205	—		
Lactobacillus arabinosus	IFO	3070	—		
Lactobacillus bulgaricus	IFO	3533	—		
Lactobacillus delbrueckii	IFO	3202	—		
Lactobacillus delbrueckii	IFO	3534	—		
Lactobacillus fermentum	IFO	3071	—		
Micrococcus aurantiacus	IFO	12422	*Mau* I	CTGCAG	isoscizomer of *Pst* I (16)
Micrococcus flavus	IFO	3242	—		
Micrococcus glutamicus	ATCC	13032	—		
Micrococcus luteus	IFO	3064	—		
Micrococcus luteus	IFO	12992	*Mlu* I	A↓CGCGT	new enzymes (4)
Micrococcus luteus	ICR	1850	—		
Micrococcus lysodeikticus	IFO	3333	—		
Micrococcus roseus	IFO	3764	—		
Micrococcus species	AKU	510	—		
Micrococcus species	AKU	520	—		
Micrococcus varians	IFO	3765	—		
Mycobacterium avicem	IFO	3082	—		
Microbacterium smegatis	IFO	3083	—		
Nocardia erythropolis	IFO	12320	—		
Nocardia erythropolis	IFO	12682	—		
Nocqrdia mexicana	IFO	3973	—		
Pediococcus homari	IFO	12218	—		
Proteus retgeri	ICR	0650	—		

Pseudomonas aeruginosa	IFO	3080	—		
Pseudomonas aeruginosa	IFO	3445	—		
Pseudomonas aeruginosa	IFO	3919	—		
Pseudomonas aeruginosa	ICR	3250	—		
Pseudomonas aeruginosa	TOYOBO		—		
Pseudomonas alkaligenes	ATCC	12815	*Pai* I	GGCC	isoschizomer of *Hae* III (17)
Pseudomonas alkanolytica	IFO	12319	*Pan* I	C↓TCGAG	isoschizomer of *Xho* I (18)
Pseudomonas convexa (Chester)	IFO	3773	—		
Pseudomonas cruciviae	ATCC	3115	—		
Pseudomonas decanhae	IAM	1048	—		
Pseudomonas destrolytica	IFO	12570	—		
Pseudomonas diminuta (Leifson)	IFO	12697	—		
Pseudomonas fluorescens	IFO	3081	—		
Pseudomonas fluorescens	IFO	3507	*Pfl* I	?	
Pseudomonas fluorescens	IAM	1057	—		
Pseudomonas groveolons (taetrolens)	IFO	3460	—		
Pseudomonas iodium	IFO	3558	—		
Pseudomonas maltophila	IFO	12690	—		
Pseudomonas marginalis	IFO	3925	—		
Pseudomonas ovalis	IFO	3738	—		
Pseudomonas ovalis					
Pseudomonas polycolor	IFO	3918	—		
Pseudomonas putida	ATCC	12533	—		
Pseudomonas putida C-83	TOYOBO		*Ppu* I	GGCC	isoschizomer of *Hae* III (17)
Pseudomonas putida C-175	TOYOBO		—		
Pseudomonas riboflavina (Foster)	IFO	3140	—		
Pseudomonas salanacerium	IFO	3509	—		
Pseudomonas striata	IFO	12996	—		
Pseudomonas stutzeri	IFO	12695	—		
Pseudomonas syncyanae	IFO	3757	—		
Pseudomonas synxantha	IFO	3757	—		
Pseudomonas species AK-LPL-7	TOYOBO		—		
Pseudomonas tobaci	IFO	3508	—		
Sarcina aurantiaca	IFO	3046	—		
Sarcina lutea	IFO	3232	—		
Sarcina lutea	IFO	1099	—		
Serratia marcescens	IFO	3046	—		
Serratia marcescens	IFO	3054	—		
Serratia plymuthium	IFO	3055	—		
Staphyrococcus aureus	IFO	3082	—		
Staphyrococcus faecalis	IFO	3181	—		
Staphyrococcus faecalis	IFO	3826	—		
Staphyrococcus lactis	IFO	2546	—		
Streptomyces adorifer	ATCC	6246	—		
Streptomyces albus	IFO	3418	—		
Streptomyces albus	IFO	3422	—		
Streptomyces albus	IFO	3710	—		

Streptomyces albus	IFO	13011	—		
Streptomyces caudidus	ATCC	13077	—		
Streptomyces cellulosae	IFO	3713	—		
Streptomyces flaveolens	IAM	0005	—		
Streptomyces griseus	IAM	0084	—		
Streptomyces intermedius	ATCC	3329	—		
Streptomyces longisporus	ATCC	14697	—		
Streptomyces microflavus	IFO	13062	—		
Streptomyces parourascens	IFO	13077	—		
Streptomyces phaeochromogenes	IFO	3108	*Spa* I	GCATGC	isoschizomer of *Sph* I (19)
Streptomyces purpeofusoeus	IFO	12905	—		
Streptomyces resistsmyceficus	ATCC	19804	—		
Streptomyces resistamyceficus	ATCC	19807	—		
Streptomyces rochei	IFO	12908	—		
Streptoverticillium netropsis	IFO	3723	—		
Streptoverticillium netropsis	IFO	12893	—		

Footnotes to Table:

1. Abbreviations for the sources of strains. AKU: Faculty of Agriculture, Kyoto University, ATCC: American Type Culture Collection, Rockville, Maryland, USA., IAM: Institute for Applied Microbiology, University of Tokyo, ICR: Institute for Chemical Research, Kyoto University, IFO: Institute for Fermentation, Osaka, NRRL: Northern Utilization Research and Development Division, US Department of Agriculture, Peoria, Illinoi, USA, TOYOBO: Toyoboseki Co.
2. Recognition sequences are written from 5′ to 3′, only one strand being given, and when known, the site of cleavage is indicated by the arrow.
3. Mode of cleavage of *Fok* I is as follows: (5′) ... GGATG NNNNNNNNN↓
   (3′) ... CCTAC NNNNNNNNNNNNN↑

this fractionation, cellular DNA and RNA and most of proteins including non-specific nucleases are recovered in the flow-through (non-adsorbed) fraction, and this would make it difficult to detect activities of specific enzymes in the fractions. It is quite likely that we are missing enzymes with poor affinity toward phospho-cellulose. Nevertheless, we could isolate eighteen species of enzymes in which four were found to be new enzymes. A common cloning vector pBR322 contains a single cutting site of *Aat*I in the upstream region of the *bla* gene, and two cutting sites of *Ban*II, but separated by fourteen base-pairs, in the *tet* gene. *Mlu*I does not cleave this plasmid. *Fok*I was a new type of enzyme which generates a protruding 5′-end of four nucleotides at the site nine nucleotides downstream from the recognition sequence. These enzymes would become powerful tools for gene engineering. The rmeaining fourteen enzymes were isoschizomers of known enzymes, but some of enzymes (*e.g. Eco*lICRI, *Ban*I, *Spa*I, *Gin*I, *Aat*I, *Mau*I and *Ggl*I) were very high in yields and could be easy to purify. Some of the originally repored enzymes may be replaced by these enzymes.

ACKNOWLEDGEMENTS

We thank Dr. H. Takahashi of Institute of Applied Microbiology, University of Tokyo, Dr. T. Tochikura of Department of Food Science and Technology, Faculty of Agriculture, Kyoto University, and Dr. K. Soda of this Institute 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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