
A new restriction endonuclease from *Spirulina platensis*

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ABSTRACT

Three restriction endonucleases, SpI, SpII and SpIII have been purified partially from *Spirulina platensis* subspecies siamese and named. SpI cleaves bacteriophage lambda DNA at one site, phi X 174 RF DNA at two sites, but does not cleave pBR322 DNA. This enzyme recognizes the sequence 5'CGTACG3' and 3'GCATCG5' cuts the site indicated by the arrows. SpII is an isoschizomer of TthIII and SpIII is an isoschizomer of HaeIII.

INTRODUCTION

Over the years, a large number of site-specific endonucleases have been isolated from a wide variety of microorganisms(1). We have been studying mass-cultivation and molecular breeding of *Spirulina*, which is a multicellular filamentous blue-green algae with a spiral shape. During our biochemical research of *Spirulina*, we found three type II restriction endonucleases. Analysis with various DNA substrates and of sequence around the restriction sites indicated that one of them, named SpI had new specificity and the other two were isoschizomers of known enzymes.

MATERIALS AND METHODS

Spirulina strain and medium: *Spirulina platensis* subspecies siamese was isolated from a salt lake in Ethiopia. It was grown at 30°C in 25 l cylindrical flasks with sterile air/CO₂(5%) bubbling from below and with illumination from four 120cm fluorescent lighting tubes placed at 10cm distance. The growth medium (2), consists of (g/l): NaHCO₃, 16.8; K₂HPO₄, 0.5; NaNO₃, 2.5; K₂SO₄, 1.0; NaCl, 1.0; MgSO₄·7H₂O, 0.2; CaCl₂·7H₂O, 0.04; FeSO₄·7H₂O, 0.01; EDTA, 0.08; traces (0.01-3 mg/l) of BO₃⁻, Mn²⁺, Zn²⁺, Cu²⁺, MoO₄⁻, VO₃⁻, Cr³⁺, Ni²⁺, Ti⁴⁺ and Co²⁺; at pH 8.6. From an inoculate of 1 l, 2 weeks were required to collect 100 g wet weight cells from 10 l medium, which were harvested and then stored at -80°C.

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DNA preparation: Bacteriophage lambda DNA was obtained from Takara Shuzo (Kyoto, Japan). Phi X 174 RF DNA was obtained from BRL (Bethesda, U.S.A.). pBR322 and pA03 were purified from cleared lysate by CsCl-EtBr equilibrium centrifugation.

Enzymes: Restriction enzymes, alkaline phosphatase and T4 polynucleotide kinase were obtained from Takara Shuzo, P1 nuclease from Yamasa Shoyu (Choshi, Japan).

Enzyme assay: Samples (1-5 μ l) of column fractions were incubated at 37°C for 1-2 hr in reaction mixture of 30 μ l containing 1 μ g DNA, 10 mM Tris·HCl, 7 mM MgCl₂, 100 mM NaCl, 2 mM 2-mercaptoethanol and 0.01% bovine serum albumin. After digestion, they were loaded onto 0.8% agarose slab gel in Tris·acetate buffer (40 mM Tris, 20 mM Na-acetate, 2 mM EDTA, pH 7.5). Electrophoresis was carried out at 100 V for 2 hr.

RESULTS

Purification of *Sp*I: Twenty grams of the frozen cells were suspended in 50 ml of buffer containing 10 mM Tris·HCl, 10 mM 2-mercaptoethanol and 0.1 mM EDTA and were disrupted by sonication (2 min). Cell debris was removed by centrifugation at 12,000 x g for 15 min at 5°C.

All subsequent steps were carried out at 0-5°C. A 25% stock solution of streptomycin sulfate was added to 2% and the mixture was kept at 0°C for 1 hr. The precipitate containing nucleic acid was removed by centrifugation (12,000 x g, 15 min), and to the supernatant was added solid ammonium sulfate to 70% saturation. The resulting precipitate was collected by centrifugation, dissolved in 10 mM Tris·HCl, 10 mM 2-mercaptoethanol, 0.1 mM EDTA and 10% glycerol, pH 7.5 (T-buffer), and dialyzed against the same buffer. The solution was applied to a DEAE-cellulose column (DE52, Whatman) and chromatographed with a linear gradient of 0-0.3 M NaCl in T-buffer to separate *Sp*I, II and III. *Sp*I activity eluted between 0.15 and 0.2 M NaCl. Fractions with *Sp*I activity were combined, dialyzed overnight against 10 mM potassium phosphate, 10 mM 2-mercaptoethanol, 0.1 mM EDTA, pH 7.0 (P-buffer). Dialyzed fractions were applied to heparin agarose column and chromatographed with a linear gradient of 0-1 M NaCl in P-buffer. *Sp*I activity eluted at approximately 0.5 M NaCl. The enzyme fractions were combined and dialyzed overnight against T-buffer containing 0.1 M NaCl. Dialyzed fractions were applied to a DEAE-cellulose column and the column was eluted with a linear gradient of 0.1-0.2 M NaCl. Fractions with enzyme activity were pooled, concentrated by dialysis against 10 mM potassium phosphate, 10 mM 2-mercaptoethanol and 50% glycerol, and bovine serum albumin was added to a concentration of 100 μ g/ml. At this

Table 1 Specificities of restriction enzymes of *S. platensis* subspecies *siamese*.

Enzymes	Numbers of cleavage sites				specificities
	pBR322	pA03	φX174 RF	lambda	
<u>SpI</u>	0	0	2	1	new enzyme
<u>SpII</u>	1		0	2	isoschizomer of <u>TthIII</u>
<u>SpIII</u>	22	4	11	50	isoschizomer of <u>HaeIII</u>

stage, phi X RF DNA digested with an excess enzyme for 24 hr gave sharp bands on agarose gels.

Recognition sequence of SpI, II and III: Viral and plasmid DNAs (pBR322, phi X 174 RF, pA03, lambda and T4dC) were digested with the respective enzyme and the resulting restriction fragments were compared with those of known enzymes. The results of analysis are summarized in Table 1. SpII and SpIII were found to be isoschizomers of TthIII from Thermus thermophilus strain 111(3) and HaeIII from Haemophilus aegyptius(4), respectively. The specificity of SpI was different from those of known enzymes, thus we further analyzed its recognition sequence.

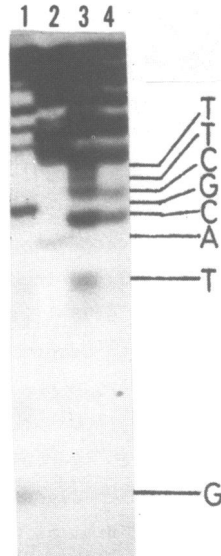


Fig.1 Autoradiogram of 20% polyacrylamide gel electrophoresis for 5'-terminal region of the 65 bp SpI-TaqI fragment of phi X 174. Fragments in lane 1,2, 3,4 were produced by chemical cleavage,G, A C, T+C, C, respectively.

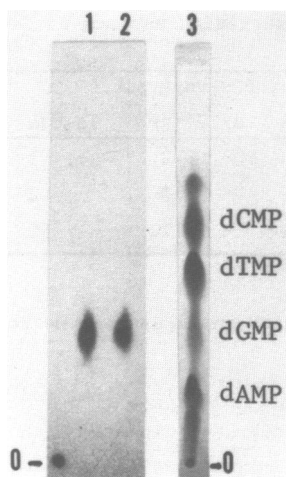


Fig.2 Identification of the 5'-nucleotide of the SplI cleaved fragments. The 5'-³²P-labelled SplI fragments of phi X 174 RF DNA were completely digested with nuclease P1, and the resulting mononucleotides were analyzed by chromatography on cellulose thin layer plate. 1, 2, Autoradiogram of ³²P-labelled 5'-mononucleotide of each SplI-TaqI fragment. 3, Photograph of authentic 5'-NMP under UV light.

Preliminary experiments with SplI on phi X 174 RF DNA and double digestion of this DNA with SplI and a variety of other restriction enzymes show that the two cleavage sites can be assigned on phi X 174 RF at the positions of about 420 and 2790. Similarly the unique SplI cutting site on lambda DNA is located at the position of about 19,000 (data not shown). A computer search through the sequences of phi X 174 RF DNA, lambda and pBR322 indicated that the hexanucleotide sequence CGTACG should be located at the positions of 413 and 2794 in phi X 174 and at the position of 19327 in lambda, and should not occur in pBR322. Thus the recognition sequence of SplI is predicted to be CGTACG.

Consequently, we used two methods to determine the recognition and cleavage specificity of SplI. At first, phi X 174 RF DNA was cleaved with SplI and two resulting fragments were dephosphorylated with bacterial alkaline phosphatase, and labelled at the 5' ends with [γ -³²P]ATP and T4 polynucleotide kinase. The labelled molecules were then digested with TaqI. The 65 bp and 250 bp labelled fragments were isolated from 5% polyacrylamide gel, and were sequenced respectively by the method of Maxam and Gilbert(5). Figure 1 shows that the 5' terminal sequence of 65 bp fragment is read out as 5' GTACG---. On the other hand the 5' terminal sequence of the 250 bp fragment was 5' GTACG-

-- (data not shown). Therefore it was indicated that the alignment of 5' terminal sequence generated by SpI cleavage was

5' GTACG---, and the site of cleavage lies C[↓]GTACG .
---GCATG 5'

To confirm this result, we analyzed 5'-labelled nucleotide of the SpI-TaqI fragments. Each 5'-labelled fragment was digested with P1 nuclease completely to 5'-mononucleotides and chromatographed on a cellulose thin layer (Funakoshi Abicel SF plate) by using saturated ammonium sulfate-0.1 N sodium acetate (pH 6.0)-isopropanol (79:19:2)(6). Non-radioactive 5'-mononucleotides were located under UV light and radioactive nucleotides were located by autoradiography. Fig. 3 shows that the labelled 5'-mononucleotide of each fragment is 5'-GMP. Thus we conclude that SpI recognizes the following hexanucleotide sequence and introduces cleavage at the indicated position,

5' C[↓]GTACG 3'
3' GCATGC 5'.
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