PURIFICATION, PROPERTIES AND RECOGNITION SEQUENCE OF SITE-SPECIFIC RESTRICTION ENDONUCLEASE FROM "ACETOBACTER LIQUEFACIENS"¹

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A type II restriction endonuclease was purified from "Acetobacter liquefaciens" IAM 1834 by consecutive column chromatography on heparin-Sepharose CL-6B, DEAE-Sepharose CL-6B and Sephacryl S-400 superfine. The purified enzyme was homogeneous on polyacrylamide gel disc electrophoresis. The enzyme preparation was essentially free from other nuclease activity, as judged by constancy of a lambda DNA-digest electrophoretic pattern after prolonged incubation for 24 hr. The enzyme was optimally active at 37° at pH 7.5, and did not require NaCl, which rather inhibited its activity. The recognition sequence for the enzyme was determined to be 5'-G-G-A-T-C-C-3', and the enzyme was found to cut between G and G in the sequence, being an isoschizomer of the endonuclease from "Bacillus amyloliquefaciens" H (Bam HI).

To date, a number of restriction endonucleases have been reported (1). These site-specific restriction endonucleases are virtually indispensable for physical mapping, DNA sequencing, gene isolation and recombinant DNA techniques. However, there have been no reports on site-specific restriction endonucleases in acetic acid bacteria.² We have been studying the biochemistry, physiology and taxonomy of these organisms, and find that many strains of Gluconobacter and Acetobacter species produce restriction endonucleases with different specificity.³ This paper describes the purification, properties and recognition sequence determination of a site-specific restriction endonuclease from "Acetobacter liquefaciens" IAM 1834.

¹ Restriction Endonuclease in Acetic Acid Bacteria. Part I. Abbreviation: Ali I, restriction endonuclease of "Acetobacter liquefaciens" IAM 1834.
² Very recently, restriction enzymes have been reported in Acetobacter aceti [H. Sugisaki, Y. Maekawa, S. Kanazawa and M. Takanami, Nucl. Acids Res., 10, 5747 (1982)].
³ Data to be published.
MATERIALS AND METHODS

Microorganism. “Acetobacter liquefaciens” IAM 1834 was used to isolate a site-specific restriction endonuclease; it was kindly supplied by Dr. K. Yamasato, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan. Since the name Acetobacter liquefaciens is not on the Approved Lists of Bacterial Names, 1980 (2), it is here enclosed in quotation marks together with other unlisted bacterial species. According to Bergey’s Manual (3), this organism is classified as Acetobacter aceti subsp. liquefaciens.

Culture of microorganism. The microorganism was grown with continuous rotary agitation in 5-l conical flasks containing 1 l of medium composed of 5 g glucose, 15 g glycerol, 5 g peptone, 1 g yeast extract and 1 g malt extract at pH 6.8 for 24 hr at 30°C. The seed cultures were grown for 48 hr at 30°C in 500-ml conical flasks containing 100 ml of the same medium. The cells were harvested by centrifugation, washed several times with 10 mM Tris-HCl (pH 7.5) and stored at -20°C. The cell yield was calculated to be about 2.0 g (wet weight) per l of cultured medium.

Assay of ammonium sulfate and column fractions. Aliquots (4.0 µl) of ammonium sulfate and column fractions at each purification step were incubated for 60 min in 20 µl reaction mixtures containing 0.5 µg lambda DNA, 7 mM 2-mercaptoethanol, 7 mM MgCl$_2$ and 10 mM Tris-HCl (pH 7.5). The enzyme reaction was stopped by adding Stop Mixture (5 µl) containing 50% glycerol, 1% sodium dodecylsulfate and 0.02% Bromophenol Blue. The reaction mixtures were then analyzed by electrophoresis on horizontal agarose gel (0.700 agarose in 0.5 µg/ml ethidium bromide, 1 mM EDTA, 36 mM Tris-HCl and 32 mM NaH$_2$PO$_4$, pH 7.8, 15x14x0.7 cm) at 0.6 V/cm for 12 hr. Ethidium bromide-stained bands of lambda DNA digests were visualized on a Model TM-15 Chromato-Vue transilluminator (Ultra-Violet Products, Inc., San Gabriel, California, U.S.A.). The developed horizontal agarose gel was photographed using Kodak technical pan film 2415 and a Kodak Wratten gelatin filter.

Digestion of DNA with restriction endonuclease. The standard digestion procedure was to incubate 1 µg lambda DNA in 20 µl AlII buffer (7 mM 2-mercaptoethanol, 7 mM MgCl$_2$ and 10 mM Tris-HCl, pH 7.5) for 60 min at 37°C with one unit of restriction endonuclease. The reaction was terminated by adding 5 µl of Stop Mixture, and the reaction mixture was analyzed by agarose gel electrophoresis as described above. One unit of enzyme activity was defined as the amount of enzyme that fully digests 1 µg lambda DNA under the above assay conditions.

Protein determination. Protein was determined by the method of LOWRY et al. (4) with crystalline bovine serum albumin as standard.

Gel disc electrophoresis. To examine the homogeneity of purified enzyme preparation, gel disc electrophoresis was carried out on 7.5% polyacrylamide gel with a Tris buffer system of pH 8.3, according to the method of DAVIS (5). About 20 µg of the purified enzyme were applied to the gel and run at a constant current of 2 mA
per tube (0.5×8 cm) for 120 min. The developed protein was stained with 0.005% Coomassie Brilliant Blue R-250.

Nucleotide sequence determination. The nucleotide sequence was analyzed by the method of Maxam and Gilbert (6). 5'-Terminal nucleotides were identified as described by Ikawa et al. (7).

Chemicals. Escherichia coli phage lambda DNA was obtained from Takara Shuzo Co., Kyoto, Japan. Phage M13 mp7 RF DNA was obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Phage ϕX174 RF, virus SV40 and plasmid pBR322 DNAs were obtained from Bethesda Research Laboratories, Inc., Gaithersburg, Maryland, U. S. A. Heparin-Sepharose CL-6B, DEAE-Sepharose CL-6B and Sephacryl S-400 superfine were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

RESULTS

Purification of Ali I endonuclease

All procedures were carried out at 4°.

Step 1. Preparation of extract and streptomycin fractionation. Cells of “Acetobacter liquefaciens” IAM 1834 (150 g, wet weight) were suspended in 1.5 l of buffer, comprised of 7 mM 2-mercaptoethanol and 10 mM Tris-HCl (pH 7.5). The suspended cells were disrupted by sonication at 180 W for 6 min. The sonicated suspension was centrifuged for 1 hr at 28,000 × g. The pellets were discarded, and the clear supernatant was adjusted to 100 mM NaCl by addition of solid NaCl. An aqueous solution of streptomycin sulfate (10% w/w) was added drop by drop to the supernatant to a final concentration of 1%. After constant stirring for an additional 30 min, the precipitate was removed by centrifugation for 15 min at 28,000 × g.

Step 2. Ammonium sulfate fractionation. Solid ammonium sulfate was added to the streptomycin supernatant (1.48 l) to give 35% concentration. The solution was left standing overnight. Then, the precipitate was separated and discarded by centrifugation. To the supernatant, ammonium sulfate was added again to produce 60% concentration. After standing overnight, the resulting precipitate was collected by centrifugation and dissolved in Buffer A (7 mM 2-mercaptoethanol, 7 mM MgCl₂, 100 mM NaCl and 100 mM Tris-HCl, pH 7.5). The solution was dialyzed against Buffer A for 1 day.

Step 3. Chromatography on heparin-Sepharose CL-6B. The dialyzed solution (49 ml) was applied to a heparin-Sepharose CL-6B column (1.7×16 cm), previously equilibrated with Buffer A. The column was washed with Buffer A. From fraction No. 107, the enzyme was eluted with a linear gradient of 0.1 to 0.6 M NaCl at the flow rate of 10 ml/hr, the total volume of the NaCl solution being 500 ml. Fractions of 5 ml were collected. A typical elution pattern is shown in Fig. 1. Endonuclease activity was recovered from fraction Nos. 120 to 156. These active
5-ml fractions were combined and concentrated with polyethylene glycol 6000. The concentrated enzyme solution was dialyzed for 1 day against Buffer B (7 mM MgCl₂ and 100 mM Tris-HCl, pH 7.5).

**Step 4. Chromatography on DEAE-Sepharose CL-6B.** The dialyzed solution (42 ml) was chromatographed on a DEAE-Sepharose CL-6B column (1.0 × 25 cm), previously equilibrated with Buffer B. The column was washed with Buffer B. From fraction No. 41, the column was developed with a linear gradient of 0 to 0.7 M NaCl (500 ml) at the same flow rate as in Step 3 (10 ml/hr) (Fig. 2). Endonuclease activity was recovered in fraction Nos. 54 to 60. These 5-ml active fractions were combined and concentrated.

**Step 5. Gel filtration on Sephacryl S-400 superfine.** The concentrated solution (6 ml) was applied to a Sephacryl S-400 superfine column (1.7 × 67 cm), previously equilibrated with Buffer C (7 mM 2-mercaptoethanol, 7 mM MgCl₂, 200 mM NaCl and 100 mM Tris-HCl, pH 7.5). The elution was run with Buffer C at the flow rate of 10 ml/hr (Fig. 3). Endonuclease activity was recovered in fraction Nos. 81 to 93. These active 2-ml fractions were collected to be concentrated with polyethylene glycol 6000.

Purification steps for the endonuclease are summarized in Table 1.

**Homogeneity of purified enzyme**

The purified enzyme was examined for homogeneity by polyacrylamide gel disc electrophoresis and found to be stained as a single band as shown in Fig. 4.

**Some properties of purified enzyme**

*Lambda DNA cleavage.* After one μg of lambda DNA was incubated for 0
to 24 hr with the purified enzyme preparation (1 unit) in reaction mixtures at pH 7.5 at 37°C, cleavage products were analyzed by horizontal agarose gel electrophoresis. As shown in Fig. 5, the lambda DNA was completely cleaved at five sites for
The prolonged incubation for 24 hr gave no change in the gel electrophoretic pattern of the cleavage products. The purified enzyme was, therefore, recognized as a site-specific endonuclease (Ali I).

**pH and temperature dependency.** The purified site-specific endonuclease was measured at different pH levels ranging from 4.5 to 10.5 and different temperatures from 0 to 60°. pH dependency was indicated by the intense enzyme activity occurring at pH 7.5 to 8.5 at 37°, as judged by the absence of partial digests of lambda DNA. The enzyme catalyzed the reaction to some extent at pH 6.5 and 9.5, but hardly at all at pH 10.5. So the optimal pH was determined to be 7.5. The optimal temperature selected for the enzyme reaction was 37°, since partial digests of

### Table 1. Purification of site-specific restriction endonuclease from “Acetobacter liquefaciens” IAM 1834.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume (ml)</th>
<th>Total protein (mg)</th>
<th>Total activity (unit)</th>
<th>Specific activity (unit/mg)</th>
<th>Recovery (%)</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptomycin</td>
<td>1,500</td>
<td>9,800</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Ammonium sulfate (35-60%)</td>
<td>49.0</td>
<td>750</td>
<td>28,800</td>
<td>38</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Heparin-Sepharose CL-6B</td>
<td>42.0</td>
<td>8.8</td>
<td>25,000</td>
<td>2,900</td>
<td>87.5</td>
<td>76</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>6.0</td>
<td>0.27</td>
<td>18,000</td>
<td>66,700</td>
<td>62.5</td>
<td>1,800</td>
</tr>
<tr>
<td>Sephacryl S-400 superfine</td>
<td>1.0</td>
<td>0.084</td>
<td>12,600</td>
<td>150,000</td>
<td>43.8</td>
<td>4,000</td>
</tr>
</tbody>
</table>

Fig. 5. Analysis of cleavage products of lambda DNA incubated for 0 to 24 hr.

The purified enzyme (1.0 unit) was incubated for different times at 37° in 20 μl reaction mixtures containing 1 μg lambda DNA, 7 mM 2-mercaptoethanol, 7 mM MgCl₂ and 10 mM Tris-HCl (pH 7.5). The reaction was terminated at the time indicated, and the reaction mixtures were analyzed as described in the text.

Fig. 6. Assay to examine sodium chloride concentration for the Ali I cleavage.

The purified enzyme (1.0 unit) was incubated at 37° for 60 min in 20 μl reaction mixtures containing 1 μg lambda DNA, 7 mM 2-mercaptoethanol, 0.1 mM EDTA and 10 mM Tris-HCl (pH 7.5). Sodium chloride was added to the reaction mixtures at final concentrations of 50 to 300 mM before incubation. The gel channels are labeled according to the respective concentrations of NaCl examined.

1 hr. The prolonged incubation for 24 hr gave no change in the gel electrophoretic pattern of the cleavage products. The purified enzyme was, therefore, recognized as a site-specific endonuclease (Ali I).
lambda DNA were not found at 25 to 40°. The enzyme activity gradually decreased below 20° and above 50°.

**pH and thermal stability.** The purified site-specific endonuclease was preincubated at 4° for 24 hr at different pH levels ranging from 4.5 to 10.5, and the remaining enzyme activity was measured at pH 7.5 for 60 min at 37°. Intense activity was observed over a relatively wide range of pH; the enzyme was stable between pH 4.5 and 9.5 under the above experimental conditions. At pH 10.5, the enzyme was nearly inactivated. The thermal stability of the purified enzyme was examined by heating to 37 to 80° for 5 min at pH 7.5. Considerable enzyme activity persisted at temperatures up to 50°, as judged by the absence of partial digests of lambda DNA; but no activity occurred above 55°.

**Effect of divalent cations on enzyme activity.** The effect of divalent cations on enzyme activity was examined in the presence of 0.1 mM EDTA. The enzyme activity was markedly stimulated by 6–8 mM MgCl₂. The stimulation with MnCl₂ was not so complete as with MgCl₂ at the same concentration; partial digests of lambda DNA were seen in the presence of MnCl₂. When neither MgCl₂ nor MnCl₂ was added to the reaction mixture, no cleavage of lambda DNA occurred. With CoCl₂, NiCl₂, CdCl₂, BaCl₂ or CuCl₂, no activity was found.

**Effect of sodium chloride on enzyme activity.** The effect of NaCl on enzyme activity was examined in the presence of 7 mM MgCl₂. As shown in Fig. 6, the purified enzyme did not require NaCl for activation, or rather was markedly inhibited by 50 to 100 mM NaCl. Little activity was found at 200 to 300 mM NaCl.

**Specificity of phage, virus and plasmid DNAs.** Several DNAs (one unit) of phage, virus and plasmid were sufficiently digested with the purified site-specific endonuclease (20 units) for 2 hr at 37° at pH 7.5. The resulting restriction patterns were analyzed as described above. M13 mp7 RF, SV40 and pBR322 DNAs were cleaved at one site into a linear molecule, respectively, however, φX174 RF DNA was not cleaved by the enzyme. Based on these digestion data and the tabularized sequencing data for sequenced DNAs by Fuchs et al. (8), the recognition sequence for the enzyme was deduced to be a palindromic hexanucleotide sequence of 5'-G-G-A-T-C-C-3'.

**Recognition sequence and cleavage site for Ali I**

As described above, pBR322 DNA was cleaved into a linear molecule by the purified site-specific endonuclease. To determine the cleavage site of the enzyme within its recognition sequence on pBR322 DNA, the linear molecule was dephosphorylated with bacterial alkaline phosphatase, and labeled at both 5'-ends with [γ-32P]ATP using T4 polynucleotide kinase. The labeled molecule was digested with Hinfl endonuclease, and two labeled fragments (256 bp and 1375 bp length) were produced. These fragments were isolated by disc electrophoresis on 5% polyacrylamide gel, and then subjected to the Maxam and Gilbert technique for DNA (6). As shown in Fig. 7, the gel pattern of the smaller fragment revealed the
sequence G-A-T-C-C-T-C-T-A- - - at the labeled 5'-end. In the parallel experiment, the sequence of the longer fragment at the labeled 5'-end was G-A-T-C-C-A-C-T-G- - -. Furthermore, the direct characterization of the 5'-terminal nucleotide of the labeled smaller fragment by complete digestion with nuclease P1 and the separation of the mononucleotide identified the labeled nucleotide as a G residue (Fig. 8). In the parallel experiment, the 5'-terminal nucleotide of the labeled longer fragment was also found to be a G residue. Therefore, the site-specific restriction
endonuclease from "Acetobacter liquefaciens" IAM 1834 recognizes the palindromic hexanucleotide sequence of 5'-G-G-A-T-C-C-3' and cuts between the two G's in the sequence, generating 5'-cohesive termini G-A-T-C-C-3'.

**DISCUSSION**

Since the original reports on the restriction enzyme of *Haemophilus influenzae* Rd (9, 10), the list of type II restriction endonucleases from other bacterial sources has grown rapidly (1). However, there are no reports on type II restriction endonucleases produced by acetic acid bacteria.

The site-specific restriction endonuclease described above was selected as one of the most stable enzymes in acetic acid bacteria. It was purified from "Acetobacter liquefaciens" IAM 1834 by consecutive column chromatography on heparin-Sepharose CL-6B, DEAE-Sepharose CL-6B and Sephacryl S-400 superfine to an approximately 4000-fold increase in specific activity over the ammonium sulfate fraction. A number of site-specific restriction endonucleases have been reported to date, however, relatively few attempts have been made to purify the enzymes to the point of a homogeneous state (11). It is noteworthy here that our enzyme preparation obtained from "Acetobacter liquefaciens" IAM 1834 was electrophoretically homogeneous. Moreover, the purified enzyme preparation was essentially free from other nuclease activity, as judged by the constancy of the lambda DNA-digest electrophoretic pattern after incubation was prolonged for 24 hr. The enzyme was seen to cleave lambda, SV40, M13 mp7 RF and pBR322 DNAs at 5, 1, 1 and 1 sites, respectively, at 37° at pH 7.5. In order to optimize the digestion condition for the purified site-specific restriction endonuclease Ali I, several factors were systematically varied. The Ali I digestion worked best at 37° in 7 mM 2-mercaptoethanol, 7 mM MgCl₂ and 10 mM Tris-HCl (pH 7.5). Additionally, the enzyme did not require NaCl characteristically for activation.

We have determined the recognition sequence for the purified site-specific restriction endonuclease Ali I and found that it cleaves the two-fold rotationally symmetric sequence 5'-G-G-A-T-C-C-3' at the position indicated by the arrows generating fragments with cohesive termini. Therefore, the site-specific restriction endonuclease from "Acetobacter liquefaciens" IAM 1834 was identified as an iso-schizomer of the endonuclease *Bam HI* from "*Bacillus amyloliquefaciens*" H (7, 12).

**REFERENCES**