Purification, Properties and Recognition Sequence
of Site-specific Restriction Endonuclease
from *Gluconobacter cerinus* IFO 3285

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A type II restriction endonuclease, designated as *GceGLI*, was purified from cells of *Gluconobacter cerinus* IFO 3285. The purified enzyme was found to be homogeneous on polyacrylamide gel disc electrophoresis. The enzyme worked best at 37°C and pH 7.5 and required 7 mM MgCl₂ and 100 mM NaCl. The purified enzyme was stable when preincubated over a pH range of 7.5 to 9.5 for 12 hr at 4°C and at a temperature range of 37 to 40°C for 5 min at pH 7.5. The enzyme was shown to cleave ϕX174 RF, SV40, pBR322, M13 mp7 RF and Ad2 DNAs at 4, 1, 0, 0, 0 and 25 or more sites, respectively, and to recognize the DNA sequence of 5'-C-C-G-C-G-G-3' and to cut between C and G on the right side of the sequence, being an isoschizomer of *SacII* of *Streptomyces achromogenes* ATCC 12767.

Restriction endonucleases have been indispensable for genetic analysis and manipulation of DNA molecules, and their occurrence is widespread in the prokaryotic kingdom. In previous studies, we purified three restriction endonucleases from acetic acid bacteria to homogeneity on polyacrylamide gel disc electrophoresis and determined their recognition sequences and their cleavage sites on DNA molecules. This paper deals with the purification and properties and recognition sequence and cleavage site determinations of a restriction endonuclease, designated as *GceGLI*, from *Gluconobacter cerinus* IFO 3285.

MATERIALS AND METHODS

Bacterial species, strain and culture conditions. Strain IFO 3285 of *Gluconobacter cerinus* (ex Asai 1935) Yamada and Akita 1984¹ was used in this experiment. The strain, which was once identified as "*G. gluconicus*", was kindly supplied by Dr. K. Imai, Institute for Fermentation, Osaka, Japan. According to Bergey's Manual 8th Edition, however, the strain is identified as *G. oxydans* subsp. *suboxydans*. Recently, it has been classified as a new species with the revived name of *G. cerinus*⁵ and not *Gluconobacter oxydans* (Henneberg 1897) De Ley 1961.⁶ Liquid cultures of the strain were aerobically grown in medium containing 0.5% glucose, 1.5% glycerol, 0.5% peptone and 0.5% yeast extract (pH 6.8) for 24 hr at 30°C.

Assay of endonuclease activity. Aliquots (5.0 µl) of ammonium sulfate and column fractions at each purification step were incubated for 60 min at 37°C in reaction mixtures of a total volume of 50 µl containing 1 µg λDNA, 10 mM Tris-HCl, 7 mM MgCl₂, 100 mM NaCl and 7 mM 2-mercaptoethanol (pH 7.5). The reaction was terminated by adding 5 µl of a stop mixture containing 1% sodium dodecylsulfate and 10 mM ethylenediaminetetraacetate (EDTA) (pH 8.0). 5 µl of a solution comprised of 0.1% bromophenol blue and 50% glycerol. Cleavage products of λDNA were analyzed by horizontal electrophoresis on 1% agarose gel in 0.5 µg/ml ethidium bromide, 2.5 mM EDTA, 89 mM Tris and 89 mM borate (pH 8.3). One unit of enzyme activity was defined as the amount of enzyme required to completely digest 1 µg λDNA in 60 min at 37°C under the assay conditions described above. Protein was determined by Lowry et al.⁹ with bovine serum albumin as a standard.

¹Restriction Endonucleases in Acetic Acid Bacteria. Part IV. For Part III, see ref. 4.
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*¹ Bacterial names in quotation marks are neither listed in the Approved Lists of Bacterial Names⁶ nor published validly in *IJSB*. 
Polyacrylamide gel disc electrophoresis. The homo-
genesis of the purified enzyme was examined by gel disc electrophoresis on 7.5% polyacrylamide gel with a Tris-
glycine buffer system (pH 8.3) by the method of Davis.10)

Nucleotide sequence and cleavage site determinations. The nucleotide sequence was determined by the procedure of Maxam and Gilbert.11) The 5'- and 3'-terminal nu-
cleotides were identified according to Ikawa et al.12)

Chemicals. Phase λ and φX174 RF DNAs, pBR322 DNA, restriction endonucleases HaeII and HaeIII, al-
kaline phosphatase (Escherichia coli C75) and phage T4 polynucleotide kinase (E. coli B) were obtained from Takara Shuzo Co., Kyoto, Japan. Staphylococcus aureus nuclease was a product of Pharmacia P-L Biochemicals Inc., Piscataway, New Jersey, U.S.A. Nuclease P1 was from Yamasa Shoyu Co., Choshi, Japan. The 3'-end labeling kit (N-4005A) was a product of Amersham International plc, Amersham, England, U.K. Phage M13 mp7 RF and virus SV40 and Ad2 DNAs were gifts from Mr. N. Maekawa, Biochemical Research Laboratories, Toyobo Co., Otsu, Japan.

RESULTS

Purification of restriction endonuclease

Preparation of cell-free extract and strepto-
mycin and ammonium sulfate fractionations. Cells of G. cerinus IFO 3285 (170 g, wet weight) were suspended in 1.8 liters of 10mM Tris-HCl and 10mM 2-mercaptoethanol (pH 7.5) and disrupted by sonication for 6 min at 180 W. The sonicated cell suspension was cen-
trifuged at 28,000 × g for 1 hr. The clear supernatant was adjusted to 100 mM NaCl with solid NaCl, and 10% streptomycin solution (w/v, 180 ml) was added drop by drop to the supernatant to a final concentration of 1% with constant stirring. After constant stirring for an additional 30 min, the resulting precipitates were removed by centrifugation at 28,000 × g for 15 min. The enzyme solution was frac-
tionated with ammonium sulfate. The active fraction precipitated at 30 ~ 60% concen-
tration was dissolved in 100 ml Buffer A (50 mM Tris-HCl, 7 mM MgCl2, 200 mM NaCl and 7 mM 2-mercaptoethanol, pH 7.5), and the solution was dialyzed for 12 hr against Buffer A.

Heparin-Sepharose CL-6B column chromato-
graphy. The dialyzed solution (100 ml) was
subjected to heparin-Sepharose CL-6B column chromatography (column size, 1.7 × 13.0 cm). The column was rinsed with Buffer A. The enzyme was eluted at fraction No. 101 with a linear gradient of 0.2 ~ 0.7 M NaCl (400 ml) at a flow rate of 20 ml/hr. Fractions of 5 ml were collected. Endonuclease activity was recovered at fraction Nos. 145 to 170. The active frac-
tions were combined, concentrated with polyethylene glycol #6,000 and then dialyzed for 12 hr against Buffer B (10 mM KH2PO4–K2HPO4, 200 mM NaCl and 7 mM 2-mercap-
toethanol, pH 7.5).

Hydroxyapatite column chromatography. The dialyzed solution (17.5 ml) was loaded onto a hydroxyapatite column (1.7 × 11.5 cm). The enzyme was eluted at fraction No. 26 with a linear gradient of 0.01 ~ 0.5 M KH2PO4–K2HPO4 (360 ml) at the same flow rate (20 ml/hr). Endonuclease activity was re-
covered at fraction Nos. 47 to 85. The com-
bined active fractions (190 ml) were concen-
trated and dialyzed for 12 hr against Buffer C (50 mM Tris-Cl and 7 mM MgCl2, pH 7.5).

DEAE-Sepharose CL-6B column chromato-
graphy. The dialyzed solution (28.0 ml) was subjected to DEAE-Sepharose CL-6B column chromatography (column size, 1.2 × 22.5 cm). The enzyme was eluted at fraction No. 28 with a linear gradient of 0 ~ 0.3 M NaCl at the same flow rate (20 ml/hr). Endonuclease activity was recovered at fraction Nos. 47 to 67. The combined active fractions were concentrated and dialyzed for 12 hr against Buffer A.

Blue Sepharose CL-6B column chromato-
graphy. The dialyzed solution (16.0 ml) was
loaded onto a blue Sepharose CL-6B column (1.2 × 17.7 cm). The enzyme was eluted at fraction No. 31 with a linear gradient of 0.2 ~ 1.2 M NaCl (400 ml) at the same flow rate (20 ml/hr). Endonuclease activity was recovered at frac-
tion Nos. 57 to 85. The combined active frac-
tions were concentrated and dialyzed for 12 hr against 10 mM Tris-HCl, 7 mM MgCl2, 7 mM 2-mercaptoethanol and 50% glycerol (pH 7.5).

The purification steps for the enzyme are summarized in Table I.
Restriction Endonuclease of *G. cerinus*

<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>1,800</td>
<td>4,030</td>
<td>—</td>
<td>—</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>104</td>
<td>2,390</td>
<td>31,200</td>
<td>13</td>
<td>84.1</td>
<td>388</td>
</tr>
<tr>
<td>Heparin-Sepharose CL-6B</td>
<td>17.5</td>
<td>5.20</td>
<td>26,250</td>
<td>5,048</td>
<td>80.9</td>
<td>408</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>28.0</td>
<td>4.76</td>
<td>19,200</td>
<td>5,363</td>
<td>61.5</td>
<td>413</td>
</tr>
<tr>
<td>DEAE-Sepharose CL-6B</td>
<td>16.0</td>
<td>3.58</td>
<td>25,250</td>
<td>5,305</td>
<td>84.1</td>
<td>388</td>
</tr>
<tr>
<td>Blue Sepharose CL-6B</td>
<td>6.5</td>
<td>1.55</td>
<td>13,000</td>
<td>8,387</td>
<td>80.9</td>
<td>408</td>
</tr>
</tbody>
</table>

Homogeneity of purified enzyme

The purified enzyme (70 µg) was examined as to its homogeneity by polyacrylamide gel disc electrophoresis. As shown in Fig. 1, the purified enzyme was stained as a single band on a developed gel disc.

Properties of purified enzyme

Lambda DNA cleavage. One µg of λDNA was incubated with the purified enzyme (50 units) for different times from 0 to 24 hr, and the resulting cleavage products were analyzed by agarose gel electrophoresis. The λDNA was completely cleaved at 4 sites in 15 min (Fig. 2). The digestion pattern did not change after prolonged incubation for 24 hr, so the purified enzyme was recognized as being free of other non-specific endonuclease activities and thus regarded as a site-specific restriction endonuclease.

Optimum reaction conditions. The purified enzyme (1 unit) was assayed at 37 °C over a pH range of 4.5 to 10.5 and found to be active at
pHs 7.5, 8.0 and 8.5. No activity was found at pHs 4.5, 5.5 and 10.5. The optimum pH selected was 7.5. The purified enzyme (1 unit) was reacted with λDNA over a temperature range of 10 to 55°C. The enzyme cleaved λDNA completely around 37°C, so the optimum temperature was determined to be 37°C.

**pH and thermal stabilities.** Aliquots (10 units) of the purified enzyme were dialyzed at 4°C for 12 hr over a pH range of 4.5 to 10.5. The dialyzed enzyme was adjusted to pH 7.5 by dialysis against 10 mM Tris-HCl, 7 mM MgCl₂, 100 mM NaCl and 7 mM 2-mercaptoethanol (pH 7.5) at 4°C for 1 hr. The remaining enzyme activity was assayed using the original 5 units of the enzyme. The enzyme was stable toward the above-mentioned treatment at pHs 7.5, 8.5 and 9.5. Aliquots (5 μl) of the enzyme were preincubated over a temperature range of 37 to 70°C for 5 min at pH 7.5 and assayed for enzyme activity. The enzyme was stable up to 40°C.

**Effects of divalent cations and sodium chloride.** The purified enzyme (5 units) was examined as to the effects of divalent cations in 50 μl reaction mixtures containing 1 μg λDNA, 10 mM Tris-HCl, 7 mM divalent cation, 1 mM EDTA, 100 mM NaCl and 7 mM 2-mercaptoethanol (pH 7.5). The enzyme essentially required MgCl₂, which could be partially replaced by MnCl₂. However, MnCl₂ showed little activity compared with MgCl₂. No activity was found with CoCl₂, NiCl₂, CuCl₂, CdCl₂ and BaCl₂. The effect of NaCl concentration on the enzyme activity was examined, and the presence of 75~100 mM NaCl gave intense activity. The enzyme activity was inhibited by 200 mM or more NaCl. The optimal concentration of NaCl was determined to be 100 mM.

**Specificity for phage, virus and plasmid DNAs.** The enzyme (10 units) was reacted with 1 μg of phage, virus and plasmid DNAs at 37°C for 2 hr, and the reaction products were analyzed by agarose gel electrophoresis. Lambda, φX174 RF and Ad2 DNAs were cleaved at 4, 1 and 25 or more sites, respectively. However, the enzyme did not cleave M13 mp7 RF, SV40 and pBR322 DNAs. Based on the above-mentioned data and the tabularized sequencing data for sequenced DNAs of Fuchs et al., the recognition sequence of the enzyme was considered to be a palindromic hexanucleotide sequence, 5'-G-C-G-C-G-C-3', 5'-C-C-G-C-G-G-3' or 5'-C-T-C-G-A-G-3'. On comparing the digests of λDNA with the enzyme with HindIII-digests of λDNA by agarose gel electrophoresis, the enzyme was assumed to recognize the palindromic hexanucleotide sequence, 5'-C-C-G-C-G-G-3'.

**Recognition sequence and cleavage site Maxam-Gilbert sequence and 5'-end determinations on φX174 RF DNA.** As described above, φX174 RF DNA was cleaved at 1 site into a linear molecule by the enzyme. To determine the cleavage site within the recognition sequence, 100 μg of φX174 RF DNA was digested with the enzyme, and the resulting linear molecule was dephosphorylated with alkaline phosphatase and labeled at the 5'-ends with [γ-32P]ATP using T4 polynucleotide kinase. The labeled linear molecule was digested with HaeIII, and the two labeled fragments were separated from each other by 5% polyacrylamide gel electrophoresis. The separated smaller (267 base pairs) and longer (1084 base pairs) fragments were sequenced by the method of Maxam and Gilbert. The gel pattern of the smaller fragment showed the sequence of G-A-T-T-G-G-T-T-T---- from the 5'-end (coordinate 2864) in the 3'-direction (coordinate 2868 and further). On the other hand, the gel pattern of the longer fragment revealed the sequence of G-C-A-T-T-T-A-G-T---- from the 5'-end (coordinate 2859) in the 3'-direction (coordinate 2855 and further). The direct characterization of 5'-terminal nucleotides on the two fragments by complete digestion with nuclease PI and separation of mononucleotides allowed identification of the labeled nucleotides as G residues.

**Maxam-Gilbert sequence and 3'-end determinations on φX174 RF DNA.** The enzyme
was assumed to cleave the palindromic sequence of 5'-'C-C-G-C-G-G-3' between G and G from the results of the above-mentioned 5'-end labeling experiment. Since the exact cleavage site was still obscure, the following 3'-end labeling experiment was done. Fifty µg of φX174 RF DNA was digested with the enzyme and labeled at the resulting 3'-ends with [α-32P]dATP (2', 3'-dideoxyadenosine-5'-[α-32P]triphosphate) using terminal transferase. The labeled linear molecule was treated with \(Hae\)II, and the two labeled fragments were separated from each other. The separated smaller (116 base pairs) and longer (1447 base pairs) fragments were sequenced by the method of Maxam and Gilbert.11] The smaller and longer fragments showed gel patterns of (A of ddATP)-C-G-C-C-T-A-A-C-C---- and (A of ddATP)-C-G-C-C-G-T-A-A-A----, respectively, from the 3'-ends (coordinates 2861 and 2862, respectively) in the 5'-directions (coordinates 2865 and 2858, respectively, and further). The sequence between coordinates 2859 and 2864 reads 5'-C-C-G-C-G-G-3' on φX174 RF DNA. The direct characterization of 3'-nucleotides on the two fragments by complete digestion with \(S.\) aureus nuclease and separation of mononucleotides allowed identification of the labeled nucleotides as C residues.

From the results obtained above, the site-specific restriction endonuclease, designated as \(Gc\)eGLI, from \(G.\) cerinus IFO 3285 was found to recognize the hexanucleotide sequence, 5'-'C-C-G-C-G-G-3' at the positions indicated by the arrows, generating cohesive dinucleotide 3'-terminal extensions. The site-specific restriction endonuclease, designated as \(Gc\)eGLI, described here has been identified as an isoschizomer of the following endonucleases; \(Sac\)II of \(Streptomyces\) aërochromogenes ATCC 12767,*2 \(Bac\)I of \(Bacillus\) acidocaldarius ATCC 27009,*3 \(Csc\)I of \(Calothrix\) scopulorum CCAP 1410/5,15) \(Eco\)I of \(Enterobacter\) cloacae DSM 30060,*4

MraI of "Micrococcus radiodurans" ATCC 13939,\textsuperscript{16} NglIII of Neisseria gonorrhoeae KH 7764-45,\textsuperscript{17} ShyI of Streptomyces hygroscopicus\textsuperscript{*5} and SsrII of Streptomyces species Stanford.\textsuperscript{18} The cleavage site of the enzyme was found to be the same as those of SacII, CscI and SsrII.

We have found isoschizomers of SacI in G. cerinus IFO 3171, 3251, 3253, 3264, 3285, 3286 and 3290. The distribution and taxonomic significance of restriction endonucleases in Gluconobacter species will be presented elsewhere.

Appendix. Just after the manuscript had been submitted, a report on a restriction endonuclease of Gluconobacter species was published [Hiraoka et al., J. Ferment. Technol., 63, 151 (1985)].

REFERENCES
