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Characterization of a Site-Specific Restriction Endonuclease from *Rhodopseudomonas sphaeroides*

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A type II restriction endonuclease, *Rsh*I, has been partially purified from photoheterotrophically grown *Rhodopseudomonas sphaeroides* strain 2.4.1. The enzyme preparation, after a single DE-52 column fractionation, is free of 5′ exonuclease and phosphatase activities but contains a trace of 3′ exonuclease activity. Based upon deoxyribonucleic acid (DNA) sequencing data in the vicinity of the enzyme-promoted cleavage of pBR322 DNA, we have concluded that *Rsh*I probably recognizes the palinodromic hexanucleotide sequence 5′-CGATCG-3′ and cleaves between the T and C. λ*c*I857 DNA contains three *Rsh*I sites, two of which lie in the replaceable region. The plasmid pBR322, which carries resistances to ampicillin and tetracycline, contains a single *Rsh*I site in the ampicillin resistance determinant. Insertion of DNA into the *Rsh*I site of pBR322 results in loss of ampicillin resistance but retention of tetracycline resistance, thereby providing a convenient screening procedure for recombinant plasmids.

Sequence-specific endonucleases are valuable tools for the elucidation of genomic organization, the physical mapping of regions involved in genetic structure and function, and the construction of chimeric plasmids for purposes of cloning. The utility of these enzymes is found in their remarkable specificity for nucleotide sequences in double-stranded DNA molecules. Since their initial discovery 10 years ago, more than 140 such enzymes have been identified (17).

We describe in this report a simple method for isolating the sequence-specific endonuclease (*Rsh*I) from photoheterotrophically grown *Rhodopseudomonas sphaeroides* strain 2.4.1. In addition, we have determined the probable *Rsh*I recognition sequence and site of cleavage. Since *Rsh*I appears to recognize a 6-base pair sequence and produces overlapping or cohesive ends, it should be especially useful for in vitro recombination experiments.

MATERIALS AND METHODS

Strains and culture conditions. *R. sphaeroides* strain 2.4.1 was obtained from W. R. Sistrom, University of Oregon. This strain was grown on succinic acid minimal media additionally supplemented with 0.2% Casamino Acids (6). Photoheterotrophic growth was maintained in completely filled, sealed vessels under saturating illumination (>600 footcandles [ca. 6,456 lx]) provided by a bank of Sylvania incandescent flood lamps. All growth was conducted at 32°C and followed turbidimetrically with a Klett-Summerson colorimeter equipped with a no. 66 filter. Cells in late-logarithmic phase (~125 Klett units ≅ 1.3×10^9 cells per ml) of growth were harvested by centrifugation in a Sharples Super Centrifuge and stored as a cell paste at −76°C.

Preparation of the *Rsh*I active fraction. Eight grams of frozen cells was thawed and washed once with column buffer (10 mM potassium phosphate, pH 7.4; 10 mM β-mercaptoethanol; 10%, vol/vol, glycerol). The washed cell pellet was resuspended in 40 ml of column buffer, and the cells were disrupted by one passage through a French pressure cell at 20,000 lb/in^2. Phenylmethylsulfonyl fluoride was added to a concentration of 0.1 mM. Unpublished experiments (Deal and Kaplan) have demonstrated that a phenylmethylsulfonyl fluoride-inhibitable protease activity is present in extracts of *R. sphaeroides*, although no specific protease effect has been detected with respect to *Rsh*I activity. The whole cells and debris were removed by centrifugation at 12,000 × *g* for 10 min. The resultant supernatant was freed of particulate material by two successive centrifugations at 150,000 × *g* for 1.5 h. All procedures were conducted at 4°C.

Freshly prepared 5% (wt/vol) streptomycin sulfate solution (0.35 volume) was added dropwise with stirring over a 30-min period to the crude soluble protein fraction described above. The suspension was stirred on ice for an additional 30 min, and the precipitate, containing the bulk of the nucleic acids, was removed by centrifugation at 12,000 × *g* for 15 min. The remaining supernatant was dialyzed overnight against column buffer.

Particulate material remaining in the dialyzed preparation was removed by centrifugation at 12,000 × *g* for 10 min. The supernatant was loaded onto a Whatman DE-52 column (1.0 by 25 cm) previously equilibrated with column buffer. The sample was washed with 2 to 3 bed volumes of column buffer, followed by a 500-ml gradient of 0.0 to 1.0 M KCl in column buffer,

and 3-ml fractions were collected. Those fractions which exhibited endonuclease activity were pooled and dialyzed (1:100) against storage buffer (25 mM potassium phosphate, pH 7.4; 10 mM β-mercaptoethanol; 0.1 mM Na_2 EDTA; 50%, vol/vol, glycerol). *Rsh*I eluted at approximately 0.05 to 0.15 M KCl, with the bulk of the exonuclease eluting at 0.5 M KCl or higher. The preparation contained no detectable 5′ exonuclease activity and only a trace amount of 3′ exonuclease activity. The *Rsh*I preparation was stored at −20°C. The approximate yield of *Rsh*I was 1,000 units per g of cells. One unit of restriction enzyme cleaves 1 μg of λ DNA in 1 h. The enzyme activity is inhibited by NaCl or KCl concentrations greater than 100 mM.

Enzymes and chemicals. *Hae*III and *Hha*I endonucleases were purchased from New England Biolabs. *Eco*RI and *Hin*dIII were isolated by the procedures of Greene et al. (5) and Old et al. (12), respectively. Phenylmethylsulfonyl fluoride was purchased from Sigma Chemical Co. Simian virus 40 form I, φX174 replicative form, and pBR322 DNAs were generous gifts of T. Spillman, K. Postle, and C. Fornari, respectively. DE-52 was purchased from Whatman Ltd.

Enzyme assay conditions and electrophoresis. *Hha*I, *Hae*III, *Eco*RI, and *Hin*dIII were assayed by previously described procedures (5, 10, 12, 18). Assays for *Rsh*I activity were carried out in reaction mixtures (50 μl) containing 1.5 μg of λcI857 DNA, 10 mM Tris-hydrochloride (pH 7.9), 6 mM $MgCl_2$, and 0.5 mM dithiothreitol. After incubation for 1 h at 37°C, reactions were terminated by addition of 10 μl of electrophoresis sample buffer (1), and electrophoresis was performed on 1% (wt/vol) horizontal agarose slab gels. Acrylamide and agarose gel electrophoreses were performed employing standard procedures (1, 15). λcI857 DNA was isolated as described previously (4).

Determination of *Rsh*I cleavage site. Determination of the *Rsh*I cleavage site was carried out by the method of McConnell et al. (9) using pBR322 DNA. *Rsh*I cleaves pBR322 at a single site (see Fig. 2); and *Hae*III fragment containing the *Rsh*I site was eluted from a polyacrylamide gel, 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and sequenced according to the procedure of Maxam and Gilbert (8). 5′ ^{32}P-labeled DNA, cleaved by *Rsh*I, was subjected to electrophoresis adjacent to the sequencing lanes.

RESULTS

Isolation of *Rsh*I. The isolation of *Rsh*I is relatively simple, involving a single DE-52 column fractionation (see Materials and Methods section). The preparation of enzyme is free of detectable 5′ exonuclease and phosphatase activities but does contain a trace of 3′ exonuclease activity (data not shown). It appears that phosphocellulose chromatography should be avoided since *Rsh*I and nuclease activities co-elute under the usual conditions employed for restriction endonuclease isolations (5, 10, 12, 17, 18). We have found that KCl or NaCl concentrations greater than 100 mM inhibit enzyme activity (data not shown). Purification procedures utilizing other chromatography systems such as heparin-agarose and ω-aminopentyl-Sepharose have not been attempted. The enzyme is active over a pH range of 6.8 to 8.5 and a temperature range of 30 to 37°C and is stable in storage buffer at −20°C for over 6 months.

Specificity of *Rsh*I. Under the reaction conditions employed, *Rsh*I did not cleave simian virus 40 form I, φX174 replicative form, or pVH 51 (mini-ColE1) DNA. The plasmid pBR322 (3) contains one site (see below), and bacteriophage λ contains three *Rsh*I cleavage sites. Figure 1 shows the sites of *Rsh*I cleavage, which were determined relative to the known *Eco*RI and *Hin*dIII sites in λcI857 DNA (11). Two of the *Rsh*I sites (54.5% λ and 73 to 74% λ) map in the "replaceable" region of λ (2), with the third site mapping in the late gene region (26 to 28%). Thus, it is possible that this enzyme may be useful for in vitro experiments using charon phages (2) or other suitable λ vectors.

Position of cleavage and probable recognition site. The cleavage and probable recognition site of *Rsh*I were determined by the method of McConnell et al. (9) using pBR322 DNA. Figure 2 shows digests of pBR322 DNA with the endonucleases *Hha*I and *Hae*III, both in the presence and in the absence of *Rsh*I. These nucleases were chosen to localize and isolate DNA carrying the *Rsh*I cleavage site for purposes of DNA sequencing.

The *Rsh*I cleavage site was determined as follows. The pBR322 *Hae*III fragment carrying the *Rsh*I site (see Fig. 2, lane b) was eluted from a polyacrylamide gel, end-labeled at the 5′ position with [γ-^{32}P]ATP and T4 polynucleotide kinase (8), and digested with *Hha*I, and the digest was subjected to polyacrylamide gel electrophoresis. Both labeled *Hae*III-*Hha*I fragments were identified by radioautography, eluted from the gel, and purified (8). Each purified fragment was either digested with *Rsh*I or directly subjected to electrophoresis to determine which fragment carried the *Rsh*I cleavage site. Once identified, this fragment was sequenced by the method of Maxam and Gilbert (8). The mobility of the *Rsh*I cleavage fragment (Fig. 3, lane e) relative to the positions of the sequencing fragments (Fig. 3, lanes a to d) indicated that the site of cleavage lies between the T and C within the palindromic sequence 5′-CGAT↓CG-3′ (see McConnell et al. [9] for a detailed description of this procedure).

Since the *Rsh*I cleavage site lies within the above palindromic sequence, the simplest interpretation is that this sequence is also the *Rsh*I recognition site. Examination of the nucleotide sequences of φX174 (14) and simian virus 40 (13)

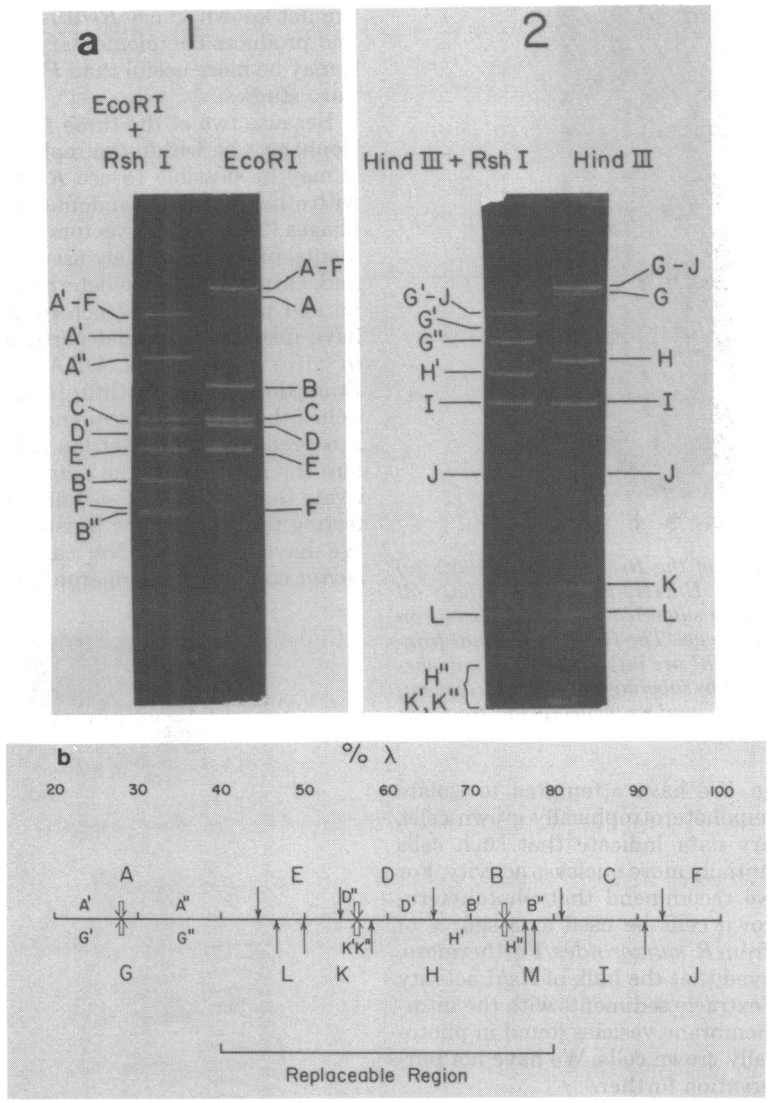

FIG. 1. *(a) Mapping of RshI sites in λcI857 DNA on 1% (wt/vol) agarose gells. RshI cleaves EcoRI fragments A, B, and D and HindIII fragments G, H, and K. Fragments produced by RshI cleavage are indicated by the ' and " superscripts. (b) Restriction map of bacteriophage λ, showing the RshI (⇕) EcoRI (↓), and HindIII (↑) cleavage sites. EcoRI fragments are labeled A to F, and HindIII sites are taken from the data of Murray and Murray (11). Fragments cleaved by RshI have been given the ' and " superscripts. Methods for determining sizes of fragments have been described previously (4, 11). The RshI sites map at approximately 26 to 28% λ, 54 to 55% λ, and 73 to 74% λ. The replaceable region of λ lies between 40% λ and 79% λ (2).*

reveals that the sequence 5'-CGATCG-3' does not occur. This is consistent with the observation that these DNAs are not cleaved by *Rsh*I.

DISCUSSION

A type II restriction endonuclease, *Rsh*I, has been partially purified from photoheterotrophically grown *R. sphaeroides*. The yields are approximately 1,000 units per g of cells when assayed at 37°C. The enzyme is sensitive to salt, and its activity is inhibited by concentrations of NaCl or KCl greater than 100 mM. The purification procedure is relatively simple, involving one column fractionation which yields a preparation sufficiently free of nucleases and phosphatase to be useful in experiments involving

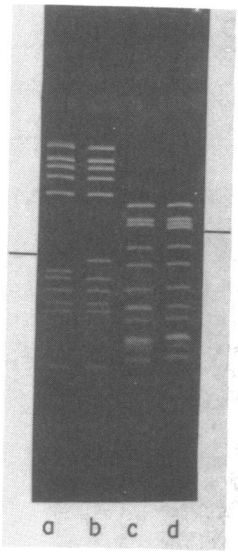

FIG. 2. *Mapping of the RshI site in pBR322. (a) RshI + HaeIII; (b) HaeIII; (c) RshI + HhaI; (d) HhaI. Samples were subjected to electrophoresis on a 5% polyacrylamide gel. The HaeIII and HhaI fragments cleaved by RshI are indicated. DNA sequencing was carried out by labeling the HaeIII fragment, cleaving with HhaI, and sequencing the fragment carrying the RshI site.*

DNA cleavage. We have attempted to isolate *Rsh*I from chemoheterotrophically grown cells, but preliminary data indicate that such cells contain substantially more nuclease activity. For this reason, we recommend that photoheterotrophically grown cells be used as a source of *Rsh*I activity from *R. sphaeroides*. Furthermore, we have observed that the bulk of *Rsh*I activity in broken cell extracts sediments with the intracytoplasmic membrane vesicles found in photoheterotrophically grown cells. We have not pursued this observation further.

With the data presently available we have concluded that *Rsh*I probably recognizes the hexanucleotide sequence 5′-CGATCG-3′. This was inferred by direct DNA sequencing across a *Rsh*I site and by a comparison of the mobility of a *Rsh*I-cleaved fragment with the sequencing fragments. There is the possibility that *Rsh*I recognizes a variant of the above sequence or a site removed from the cleavage site. Further sequencing experiments on other *Rsh*I sites are necessary to distinguish between these possibilities.

Proteus vulgaris (*Pvu*I) and *Xanthomonas nigromaculans* (*Xni*I) apparently produce isoschizomers of *Rsh*I (R. Roberts and T. Gingeras, personal communication), but the exact sites of cleavage within or near the recognition sequence are not known. Since *Rsh*I is simple to prepare and produces complementary or cohesive ends, it may be more useful than *Pvu*I and *Xni*I for in vitro studies.

Because two of the three *Rsh*I sites in bacteriophage λ lie within the replaceable region (2), it may be possible to use *Rsh*I in conjunction with other restriction endonucleases with charon phages (2) or other λ vectors for in vitro experiments. pBR322, a widely used, amplifiable plasmid, carries resistance determinants to ampicillin and tetracycline, which by proper selection have made this plasmid particularly useful for in vitro recombinant DNA experiments (3). From the data of Sutcliffe (16), who has determined the nucleotide sequence of the *amp* gene, it is predicted that insertion of DNA fragments into the *Rsh*I site of the *amp* gene should inactivate the ampicillin resistance gene without affecting the tetracycline resistance determinant. We have inserted DNA carrying the *Escherichia coli* threonine operon into the *Rsh*I site

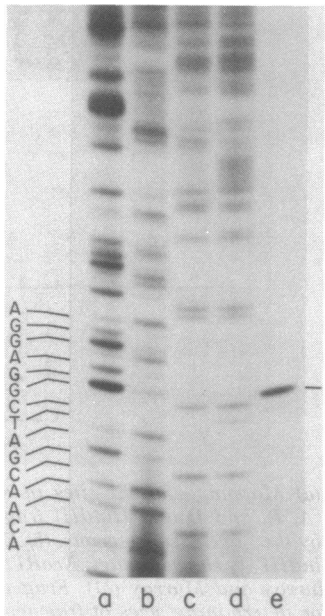

FIG. 3. *Determination of the RshI site. The HaeIII-HhaI ^{32}P-labeled fragment described in the text was sequenced by the method of Maxam and Gilbert (8). Fragments in lanes a, b, c, and d show cleavage products from the G > A, A > G, C, and C + T reactions, respectively. Lane e contains labeled DNA cleaved by RshI. The mobility of the RshI-cleaved fragment (between the C and G of the sequence CGATCG) indicates that the site of cleavage lies between the C and T of the sequence (9). The minor RshI fragment is caused by a 3′ exonuclease contaminant.*

of pBR322 (7); as predicted, all resultant Thr^+ transformants became tetracycline resistant but were sensitive to ampicillin (J. Gardner, unpublished results). Thus, *Rsh*I should be a useful enzyme for in vitro experiments using pBR322 as the plasmid vector.

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