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Partial Characterization of a DNA Restriction Endonuclease from *Ruminococcus flavefaciens* FD-1 and Its Inhibition by Site-Specific Adenine Methylation

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The principal DNA restriction-modification system of the cellulolytic ruminal bacterium *Ruminococcus flavefaciens* FD-1 is described. The restriction endonuclease *Rfl*FI could be separated from cell extracts by phosphocellulose and heparin-sepharose chromatography. Restriction enzyme digests utilizing *Rfl*FI alone or in combination with *Sal*I, a restriction enzyme isolated from *Streptomyces albus* G, showed that the DNA sequence recognized by *Rfl*FI either overlapped or was the same as that recognized by *Sal*I. DNA sequence analysis confirmed that *Rfl*FI was identical in activity to *Sal*I, with the recognition sequence being 5′-GTCGAC-3′ and cleavage occurring between G and T. Adenine methylation within this sequence can be catalyzed in vitro by *Taq*I methylase, and this inhibited the cleavage of plasmid DNA molecules by *Rfl*FI and *Sal*I. Chromosomal DNA from *R. flavefaciens* FD-1 is also methylated within this DNA sequence because neither restriction endonuclease could degrade this DNA substrate. These findings provide a means to protect plasmid molecules from degradation prior to gene transfer experiments with *R. flavefaciens* FD-1.

The ruminal bacterial species *Ruminococcus flavefaciens* plays a vital role in the conversion of plant cell wall carbohydrates into readily utilizable substrates. The ability of this species to degrade cellulose and heteroxylans has prompted research to understand the genetics and molecular biology possessed by the species to perform such processes. The strain chosen for study in our laboratory, *R. flavefaciens* FD-1, is one of the most active degraders of cellulose and has already been utilized to provide some of the genetic information currently available for *Ruminococcus* spp. (for examples, see references 3 and 17). However, the knowledge so far has been obtained from cloning strategies and expression of the gene products via *Escherichia coli* plasmid and bacteriophage λ systems (10). One objective vital for future research is the establishment of a stable DNA transfer system between ruminal bacteria and either *E. coli* or a gram-positive bacterium with a well-studied genetic system. The introduction of plasmid DNA into ruminal bacteria has been only a recent accomplishment (6–8, 15) and except in one case (16) has made use of conjugative mating procedures. Conjugal DNA transfer in *Ruminococcus* spp. has not been demonstrated, and electroporation probably offers the most effective means of establishing DNA transfer. However, despite the use of a number of cultural and enzymatic manipulations of *R. flavefaciens* FD-1 cells, electroporation has yet to result in the isolation of genotypic transformants (12a). One important factor possibly affecting the viability and expression of double-stranded DNA in ruminal bacteria is host restriction-modification systems, especially if the capsular and cell wall structures limit DNA entry. Indeed, restriction-modification systems have been shown to affect DNA transfer in other bacteria. For instance, Miller et al. (12) attributed their successful use of electroporation with *Campylobacter jejuni* to the ability to isolate plasmid DNA directly from this strain. The DNA was suitably protected from the recipient's restriction enzymes, and the transformation efficiency was 10^4-fold higher than the transformation efficiency in similar experiments using the same plasmid DNA molecule isolated from *E. coli* HB101.

The aim of the research presented here was to adequately describe the principal restriction-modification system present in *R. flavefaciens* FD-1 and to develop in vitro strategies which may be used to protect plasmid DNA prior to electroporation.

MATERIALS AND METHODS

Bacterium and growth, cell fractionation, and recovery of restriction enzymes. *R. flavefaciens* FD-1 was from our culture collection and was grown in batch cultures of EM medium (5). The cell extract (CE) used for phosphocellulose chromatography was prepared from 1.5 liters of cells grown to early stationary phase; 6 liters of cells in the mid-log phase of growth was used to produce the CE subjected to heparin-Sepharose chromatography. Cells were harvested by centrifugation at 11,300 × *g* for 10 min at 4°C. The pellets were washed twice with 500-ml volumes of TES (30 mM Tris-HCl [pH 8.0], 5 mM Na_2-EDTA, 50 mM NaCl [4°C]) buffer and then suspended in 2 ml of chromatography running buffer (20 mM Tris-HCl [pH 7.4], 7 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride [4°C]). The bacterial cells were disrupted by two passages through a French pressure cell at 21,440 lb/in^2 (American Instrument Company, Silver Spring, Md.). The crude cell lysates were further treated by either of two methods, depending on the fractionation protocol. For phosphocellulose chromatography, 60 μg of heat-treated RNase A was added to the crude cell lysate; the suspension was then centrifuged at 254,000 × *g* for 30 min at 4°C, and the supernatant fluid was harvested. For heparin-Sepharose chromatography, the crude cell lysate was treated by the procedures described by Bickle et al. (4). Both CEs were dialyzed overnight at 4°C against 3 liters of the chromatography running buffer and then stored frozen at −70°C prior to chromatography.

Chromatographic separation of cytoplasmic proteins. All

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chromatography procedures were performed at 4°C. The resins (Whatman P-11 phosphocellulose [Whatman Biosystems Ltd., Maidstone, England] and heparin-Sepharose CL-6B [Pharmacia LKB, Uppsala, Sweden]) were activated and equilibrated as recommended by the manufacturer and then packed within glass columns (7 by 150 mm). The settled bed volumes were 4.5 ml, and the flow rate of the running buffer was controlled by a peristaltic pump fitted to the outflow end of the column and attached to an automated fraction collector, increasing the void volume to 5.5 ml. The dialyzed CEs were loaded and washed over the columns with 10 ml of running buffer. A linear gradient between 0 and 0.5 M KCl was developed in 50 ml of the same buffer when phosphocellulose chromatography resin was used, and then another 10 ml of 0.5 M KCl in running buffer was used to wash the column. A similar procedure was followed for heparin-Sepharose chromatography, with the exception that the linear salt gradient was 0 to 1 M NaCl. Fractions (0.5 ml) were collected and mixed with an equal volume of sterile glycerol and then stored at −70°C until used for analysis.

Assays for restriction endonuclease activity and in vitro methylation reactions. Initially, 10-μl aliquots of the chromatographic fractions were incubated overnight at 37°C with 0.25-μg of unmethylated bacteriophage λ DNA (Boehringer Mannheim, Indianapolis, Ind.) in a reaction mixture containing 10 mM Tris-HCl (pH 7.2), 5 mM $MgCl_2$, and 70 mM NaCl. The reactions were stopped by the addition of a stop mix (11), and following electrophoresis of the DNA through 0.7% (wt/vol) agarose gels, the banding patterns were visualized by ethidium bromide staining and UV illumination. The conditions described above were found to provide optimal enzyme activity by comparative analysis with commercially available reaction buffers. The *Taq*I methylase was obtained from New England Biolabs (Beverly, Mass.). Reactions were performed according to the manufacturer's specifications and allowed to proceed overnight.

DNA sequencing of the recognition and cleavage sites for *Rfl*FI. The procedure employed was based upon that described by Lunnen et al. (9). The M13 dideoxy sequencing kit was used (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) and standard dideoxy sequencing reactions were performed with both M13 mp18 (+) DNA as a template and the 17-bp primer, according to the manufacturer's specifications. In conjunction with the four dideoxy sequencing reactions, a fifth nonterminating reaction was also performed. This nonterminating reaction generates a double-stranded substrate capable of digestion by the restriction endonuclease. The Klenow fragment was heat inactivated in this reaction; the mixture was brought up to 50 μl with the appropriate reaction buffer and restriction enzyme and then incubated overnight at 37°C. Proteins were removed by three phenol-chloroform-isoamylalcohol extractions, and any residual phenol was removed by three ether extractions. The DNA was ethanol precipitated, washed twice with 70% (vol/vol) ethanol to remove residual salts, resuspended in a minimal volume of the polymerase reaction buffer, and then divided into two aliquots. The sequencing stop mix was added directly to one aliquot, and the second aliquot was reincubated with Klenow fragment and deoxynucleoside triphosphates, following the manufacturer's specifications for the formation of blunt ends following restriction enzyme digestion. These two aliquots identify the position of DNA cleavage plus the length and direction of the overhang, respectively. Six reaction mixtures were loaded on 8% (wt/vol) acrylamide sequencing gels and then exposed to X-ray film by standard procedures (11).

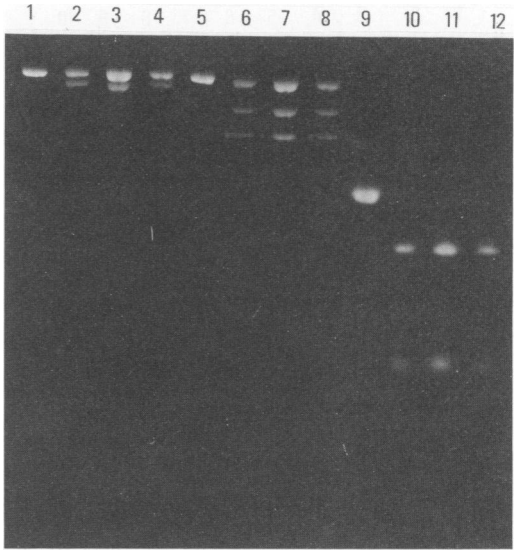

FIG. 1. Evidence demonstrating that *Sal*I and *Rfl*FI recognize the same DNA sequence. Lanes: 1, λ DNA alone; 2 through 4, λ DNA digested with *Sal*I alone, *Rfl*FI alone, and *Sal*I plus *Rfl*FI; 5, adenovirus type 2 DNA alone; 6 through 8, adenovirus type 2 DNA digested with *Sal*I alone, *Rfl*FI alone, and *Sal*I plus *Rfl*FI, respectively; 9, *Pst*I-linearized pBR322 DNA alone; 10 through 12, linearized pBR322 DNA digested with *Sal*I alone, *Rfl*FI alone, and *Sal*I plus *Rfl*FI, respectively.

RESULTS AND DISCUSSION

Elution profile for the principal restriction endonuclease activity pool from *R. flavefaciens* FD-1. The DNA fragment patterns generated from fractions of the CE of *R. flavefaciens* FD-1 indicated that two endonucleases were present. The first of these activity pools, referred to here as *Rfl*FI, was composed of fractions eluted from phosphocellulose chromatography resin by 65 to 90 mM K^+ and from heparin-Sepharose resin by 120 to 180 mM Na^+. Aliquots (1, 2, 5, and 10 μl) of the *Rfl*FI activity pool were incubated with 1 μg of unmethylated λ DNA for 16 h at 37°C. As little as 1 μl of the *Rfl*FI activity pool provided complete digestion of λ DNA in 16 h, and thus, routine enzyme digests were at least 10-fold in excess of the minimum activity required. The existence of second, less active restriction endonuclease, *Rfl*FII, was subsequently confirmed, and its characteristics are the subject of a separate article. The highest yields of *Rfl*FI activity were derived from cultures harvested during mid-log phase, and older cultures also possessed random nuclease activity which contaminated the *Rfl*FI activity pool. In previous electrotransformation experiments, cells were allowed to reach the stationary growth phase, with the objective of creating some weakening in the cell wall structure. Such a strategy proved to be successful with *Clostridium perfringens* (1) but does not seem to be applicable to members of the genus *Ruminococcus*.

Restriction enzyme mapping and in vitro methylation reactions illustrated that *Rfl*FI sites overlap *Sal*I recognition sites. Multiple restriction enzyme digests of unmethylated λ DNA and adenovirus type 2 DNA located the *Rfl*FI sites in close proximity to the *Sal*I sites (data not shown). Thus, bacteriophage λ, adenovirus type 2 DNA and linearized pBR322 DNA were digested with either *Sal*I or *Rfl*FI or both *Sal*I and *Rfl*FI. The results of these digests are illustrated in Fig. 1.

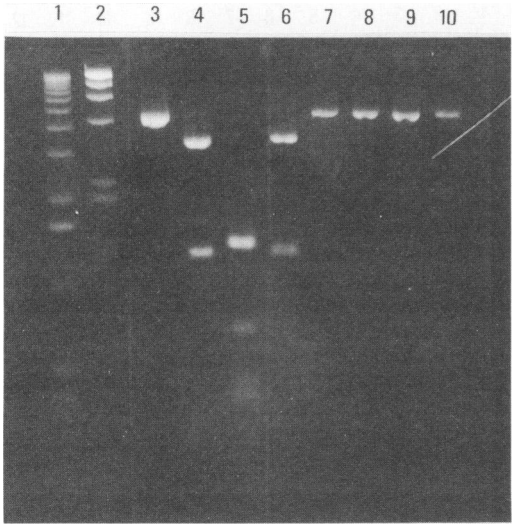

FIG. 2. Inhibition of *Rfl*FI activity by prior incubation of pBR322 DNA with *Taq*I methylase. Lanes: 1, 1-kb standard ladder; 2, λ DNA digested with *Hin*dIII; 3, unmethylated pBR322 DNA linearized with *Sca*I; 4 through 6, unmethylated, linearized pBR322, digested with *Sal*I, *Taq*I, and *Rfl*FI, respectively; 7, *Taq*I-methylated pBR322 DNA; 8 through 10, *Taq*I-methylated, linearized pBR322, digested with *Sal*I, *Taq*I, and *Rfl*FI, respectively.

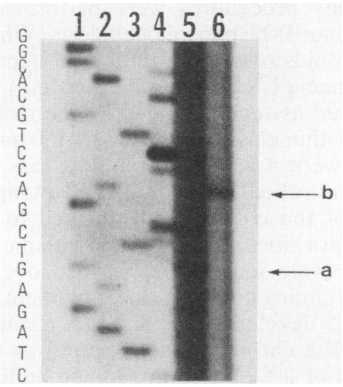

FIG. 3. DNA sequencing of the recognition site, position of cleavage, and length of nucleotide extension, specific for the restriction endonuclease *Rfl*FI. Lanes: 1 through 4, terminating sequencing reactions with ddGTP, ddATP, ddTTP, and ddCTP, respectively. 5, nonterminating reaction following digestion with *Rfl*FI, and arrow a shows the site of cleavage; 6, the length of band shifting (arrow b) following the reincubation of *Rfl*FI-cut DNA with Klenow fragment. The results described above indicate that *Rfl*FI recognizes and cleaves the following sequence:

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    ▼
5'....GTCGAC....3'
3'....CAGCTG....5'
        ▲
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After linearization, a single *Rfl*FI site was found in pBR322 DNA, and further, it was apparent that *Rfl*FI probably recognized the same DNA sequence as *Sal*I.

The *Sal*I restriction endonuclease recognizes the sequence 5′-GTCGAC-3′ (2), and the internal tetranucleotide sequence 5′-TCGA*-3′ (the asterisk [*] indicates site of methylation) is also recognized by the *Taq*I endonuclease and methylase enzymes. To determine whether *Rfl*FI activity could be inhibited by the *Taq*I methylase, pBR322 DNA was first linearized with *Sca*I and precipitated with isopropanol, and then one-half was incubated with *Taq*I methylase overnight. The methylated and unmethylated DNA substrates were then incubated overnight with either *Taq*I, *Sal*I, or *Rfl*FI. The results of these experiments are shown in Fig. 2. Methylation of pBR322 by the *Taq*I methylase sufficiently inhibited both *Sal*I and *Rfl*FI activity, as well as *Taq*I activity. Thus, adenine methylation inhibited both restriction endonucleases possessing the larger recognition sequence. Furthermore, the *Sal*I restriction enzyme was found to possess no activity against chromosomal DNA derived from *R. flavefaciens* FD-1; this is indicative of the methylation site for the cognate methylase of *Rfl*FI, which also resides within this hexanucleotide DNA sequence (data not shown).

DNA sequencing confirmed that *Rfl*FI is a true isoschizomer of *Sal*I. DNA sequencing provided definitive evidence that *Rfl*FI behaves identically to *Sal*I: the cut site is located to the 3′ side of the first guanine residue in the sequence 5′-GTCGAC-3′ (Fig. 3, lane 5, arrow a), and generates a 4-bp 5′-to-3′ overhang. This is demonstrated by the length of the band shift following reincubation of the DNA substrate with Klenow fragment (Fig. 3, lane 6, arrow b).

DNA sequencing of the cut site and overhang generated by *Rfl*FI conclusively demonstrated that this enzyme is a true isoschizomer of *Sal*I and that adenine methylation by *Taq*I methylase effectively blocks DNA cleavage by the enzyme. There have been no fewer than 11 observations of *Sal*I isoschizomers, including *Rfl*FI (14), and when the cut site has been determined, the location is identical. Both adenine and cytosine methylations within the *Sal*I recognition sequence have previously been shown to inhibit DNA cleavage by this enzyme (13); therefore, we cannot determine from these experiments which base is modified by the cognate methylase of *Rfl*FI.

The findings presented here are the first of their kind for *Ruminococcus* spp. and demonstrate that these microorganisms possess DNA-cleaving enzymes with a high level of specificity. The studies also achieved our objective: to determine how to protect plasmid DNA, prior to electroporation, from the principal restriction endonuclease present in this important ruminal bacterium. Experimentation is now planned to assess DNA entry following electroporation and phenotypic expression of in vitro methylated plasmid molecules, which are resistant to restriction enzyme activity.

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