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Isolation of Plasmid DNA from *Butyrivibrio fibrisolvens*†

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A procedure based on successive precipitation of cell lysates with sodium dodecyl sulfate-NaCl and polyethylene glycol 6000 was developed which allows the isolation of plasmid DNA from *Butyrivibrio fibrisolvens*. A survey of *B. fibrisolvens* strains isolated from the bovine rumen showed that plasmids are a common feature of this species.

The bacterial population of the rumen represents a highly competitive, densely populated microbial ecosystem, with a population density of from 10^{10} to 10^{11} cells per ml. Although the rumen microbial population has been the subject of considerable study (11, 15), no studies on the occurrence or ecological significance of plasmid-determined traits among the rumen bacteria have been reported.

Plasmids appear to be a ubiquitous feature of most naturally occurring bacterial populations. Plasmid-encoded functions generally appear to confer specific competitive advantages upon the host organism (18), and it might be expected that the rumen environment, with its rapid turnover, high population density, and varied input of organic materials, would offer many opportunities for the development of plasmid-specified functions.

Our aim was to develop methods suitable for the study of the possible role of plasmids in the adaptation of one of the predominant rumen bacteria, *Butyrivibrio fibrisolvens*, to the rumen environment. A method was required which would allow the isolation of all plasmids present regardless of size and copy number and be effective with both stock laboratory strains and new isolates from the rumen. The method described here is suitable for processing cultures of from 5 ml to several liters and provides an excellent yield of plasmid DNA. The presence of plasmid DNA was demonstrated in all strains of *B. fibrisolvens* examined.

MATERIALS AND METHODS

Media and growth conditions. The anaerobic techniques used here were based on those of Hungate (14) as modified by Bryant and Burkey (3). For the isolation of new strains, we used an anaerobic hood (1) with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. The growth media used were the rumen fluid-containing medium 98-5 (4) and the defined medium 10 (6). Medium 10 was modified by the inclusion of a B-vitamin mixture, menadione, and ferrous sulfate (22) and by substitution of the volatile fatty acid mixture of Varel et al. (21) for the mixture of Caldwell and Bryant (6). Both medium 98-5 and medium 10 were modified for use in the anaerobic hood by reduction of the Na_2CO_3 level in the medium from 0.4 to 0.0375%. The final pH of the media was 6.9 after equilibration with the gas phase in the anaerobic hood. For the preparation of petri plates or roll bottles, 2% agar was added to the media before autoclaving.

Isolation of bacteria. The standard laboratory stock strain used was *B. fibrisolvens* ATCC 19171. For the isolation of new strains, samples of rumen fluid were obtained from fistulated Holstein cows and transported to the laboratory under anaerobic conditions. The rumen fluid samples were streaked out directly in the anaerobic hood on plates of medium 10 containing either 0.2% mannitol or 0.2% xylan as the sole carbohydrate source, and the plates were incubated for 1 to 2 days at 39°C. Single colonies were transferred into medium 10 containing glucose and examined by phase-contrast microscopy after overnight growth at 39°C. Isolates with normal *B. fibrisolvens* morphology were further characterized by testing for the ability to ferment esculin, mannitol, mannose, starch, cellulose, and xylose, H_2S production, motility, gas production, Gram reaction, and volatile fatty acid production (11). Approximately 20% of the colonies originally isolated from rumen fluid were identified as *B. fibrisolvens*. Their identity was confirmed by comparing the electrophoretic pattern of cell lysates with the stock strain (ATCC 19171) by the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis system of Laemmli and Favre (16).

Isolation of plasmid DNA. Unless otherwise specified, all operations were carried out at 4°C. The cells were harvested at a cell density of 2×10^8 to 4×10^8/ml by centrifugation (Sorvall centrifuge, GSA rotor) for 20 min at 10,400 × *g*. The pellet was suspended in E buffer (40 mM Tris acetate, 20 mM sodium acetate, 5 mM EDTA) at pH 8.2 (10) to give a final volume of 1/20 the original culture volume. An equal volume of E buffer containing 2 mg of lysozyme per ml was added, and the suspension was warmed to 37°C for 5 min. A 5% (wt/vol) solution of sodium dodecyl sulfate in E buffer was added to give a final sodium dodecyl sulfate concentration of 1%, and the suspension was mixed by gentle inversion. NaCl was then added to a final concentration of 1 M, and the lysate was held on ice

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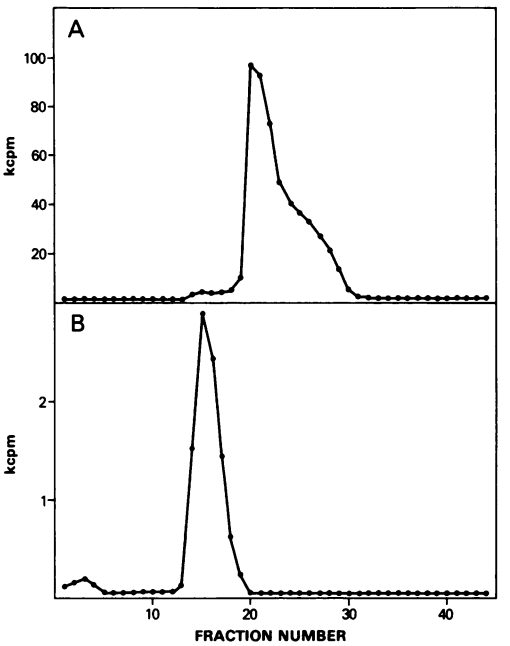

FIG. 1. Ethidium bromide-CsCl equilibrium density gradients of ATCC 19171 [^{3}H]DNA. A 10-ml culture was labeled with [6-^{3}H]thymidine, divided into two 5-ml samples, and lysed as described in the text. The preparations were brought to a final volume of 5 ml with TEN buffer, and 4.71 g of CsCl and 0.2 ml of ethidium bromide (10 mg/ml in water) were added. The samples were centrifuged for 40 h at 36,000 rpm at 15°C in a Beckman 50 Ti rotor. (A) Crude lysate; (B) lysate after NaCl and polyethylene glycol 6000 precipitation steps.

for at least 4 h to allow the precipitation of sodium dodecyl sulfate and chromosomal DNA (9). The lysate was then centrifuged for 30 min at 17,400 × *g* (Sorvall centrifuge, SS34 rotor). Plasmid DNA was precipitated from the supernatant with 10% (wt/vol) Carbowax 6000 (polyethylene glycol 6000; Union Carbide Corp.) as described by Humphreys et al. (13). The pellet containing the plasmid DNA was dissolved in 5 ml of TEN buffer (50 mM Tris-hydrochloride, 50 mM NaCl, 5 mM EDTA; pH 8.0). Plasmid DNA was isolated from this solution by dye-buoyant density equilibrium centrifugation (7) and further purified as described by Teather et al. (19).

Agarose gel electrophoresis. Agarose gel electrophoresis was carried out in a horizontal slab gel apparatus (40 by 19 cm) at 2 V/cm for 16 to 18 h with E buffer (10). Ethidium bromide (0.5 μg/ml) was included in the gel buffer.

Labeling of plasmid DNA. An overnight culture of *B. fibrisolvens* was diluted 1/20 into medium 10 containing 250 μg of deoxyadenosine per ml. After incubation for 2 h at 39°C, [6-^{3}H]thymidine (2 Ci/mmol; Amersham Corp.) was added to give 2 μCi/ml of culture medium, and incubation was continued for a further 3 h at 39°C. The cells were harvested by centrifugation, washed once with E buffer, and lysed as described above.

RESULTS

Initial attempts to demonstrate the presence of plasmid DNA with rapid screening methods based on techniques developed for other bacterial species (2, 8, 20) were unsuccessful, as was the cleared-lysate technique of Clewell (7). The protocol that was developed reflects the need to lyse the cells at a relatively low cell density (< 10^{10}/ml) to obtain a good yield of the rather large plasmids present.

A band of supercoiled DNA was identifiable in crude lysates of *B. fibrisolvens* ATCC 19171 (Fig. 1A, fractions 14 to 17). The plasmid band represented only about 2 to 3% of the total labeled material on the gradient. Figure 1B shows the purified plasmid fraction prepared from the same labeled culture after the NaCl and polyethylene glycol precipitation steps. The yield from this was similar to the apparent fraction of plasmid DNA yielded by the crude lysate (4% of the total incorporated label). Yields from larger-scale preparations were typically from 200 to 300 μg of DNA per liter of culture.

Agarose gel analysis of the purified plasmid DNA suggested the presence of a single very large plasmid in ATCC 19171. Plasmid DNA from six of the newly isolated strains of *B. fibrisolvens* (OR379, OR381, OR383, OR391, OR397, OR399) was prepared and examined by agarose gel electrophoresis. All of the strains apparently contained a similar large plasmid with a molecular weight too large for accurate measurement in the agarose gel. Heating the DNA preparations to 60°C or gentle shearing resulted in failure of the DNA to penetrate the agarose gel.

Analysis of these DNA preparations by the neutral sucrose density gradient technique of Hughes and Meynell (12) showed a single band (s^0 = 100, with RP4 DNA used as a standard). This corresponds to a molecular weight of 69 × 10^6 for covalently closed circular DNA. However, heating the DNA samples to 60°C failed to alter the sedimentation rate, and DNA isolated from the gradients failed to penetrate a 0.8% agarose gel. It was concluded that the 100 s^0 band consisted of open circular DNA and that the molecular weight of the plasmids was therefore approximately 250 × 10^6. Conversion of the covalently closed circular DNA which was initially isolated to the open circular form apparently occurs both during the final purification steps (after the polyethylene glycol 6000 precipitation) and to some extent when the sample is frozen for storage and subsequently thawed.

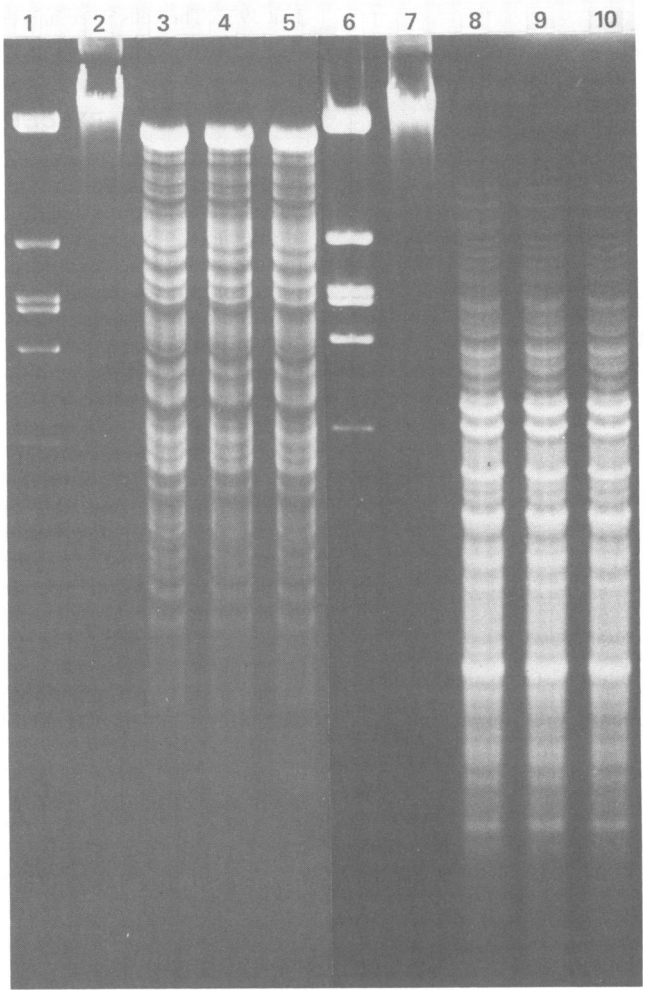

FIG. 2. Restriction enzyme digestion of ATCC 19171 plasmid DNA. The reaction mixtures each contained 18 μg of DNA in a final volume of 200 μl and were incubated at 37°C. For *Eco*RI digestion, the reaction mixture contained 100 mM Tris-hydrochloride (pH 7.6), 50 mM NaCl, 5 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, and 50 U of *Eco*RI (Bethesda Research Laboratories). For *Hind*III digestion, the reaction mixture contained 10 mM Tris-hydrochloride (pH 7.4), 60 mM NaCl, 7 mM $MgCl_2$, 0.1 mg of bovine serum albumin per ml, and 100 U of *Hind*III (Bethesda Research Laboratories). Samples were removed at zero time (15 μl) and at 30, 60, and 120 min (50 μl), and the reaction was stopped by heating the samples to 65°C for 5 min. A 1/10 volume of 50% glycerol–0.05% bromophenol blue was added, the samples were applied to a 1% agarose gel, and electrophoresis was carried out for 18 h at 1.25 V/cm. Lanes: 2 to 5, *Eco*RI digest, sampled at 0, 30, 60, and 120 min, respectively; 7 to 10, *Hind*III digest, sampled at 0, 30, 60, and 120 min, respectively; 1 and 6, *Eco*RI digest of bacteriophage λ DNA.

Agarose gel analysis of restriction enzyme digests of the plasmid DNA preparations confirmed the conclusion that the molecular weight of the plasmids was on the order of 250×10^6. Exhaustive digestion with *Eco*RI (Fig. 2) produced many fragments too large to be resolved in the agarose gel (>20 kilobase pairs) and relatively few small fragments (<2 kilobase pairs), indicating that either the frequency of the *Eco*RI recognition sequence is unusually low in these plasmids or there may be some base modification in this species which results in the protection of potential *Eco*RI sites from digestion. Digestion with *Hind*III, on the other hand, produced a more normal range of fragment sizes (Fig. 2 and 3). The sum of fragment sizes for a *Hind*III digest of the plasmid from ATCC 19171 is $>200 \times 10^6$ daltons.

Although all of the plasmids isolated were similar in size, they were not identical in that

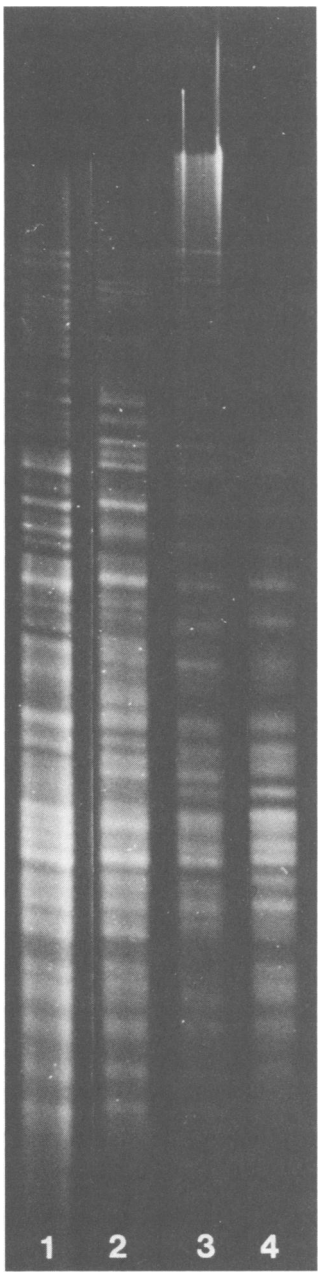

FIG. 3. *Hind*III restriction enzyme digestion of plasmid DNAs. Five micrograms of each plasmid DNA was digested for 60 min with 10 U of *Hind*III in a final volume of 50 μl of the buffer described in the legend to Fig. 2. Plasmid DNA from (1) ATCC 19171; (2) OR383; (3) OR391; and (4) OR399. Agarose concentration, 0.8%.

there were minor variations in the *Hind*III digestion pattern (Fig. 3).

DISCUSSION

Our results clearly establish the presence of plasmid DNA in *B. fibrisolvens*. All strains examined by the method described above gave essentially the same yield of plasmid DNA, and all of the plasmids isolated were very large (approximate molecular weight, 250×10^6). Plasmid DNA from both ATCC 19171 and the newly isolated strains showed only limited digestion by *Eco*RI, suggesting that there may be modification of the DNA of this species. However, experiments in this laboratory have found no evidence for the presence of either restriction enzymes or any periplasmic nuclease capable of degrading bacteriophage λ or pBR322 DNA.

The functions specified by plasmids in *B. fibrisolvens* in the rumen environment are not known, but the observation that all of the isolates examined contained a closely related plasmid suggests that the role of the plasmid is important in determining the competitive fitness of the host cell. An extensive effort has been made to isolate strains which lack a plasmid, both by screening new isolates and by using techniques commonly employed to cure plasmid-carrying strains (e.g., growth at elevated temperatures and use of chemical agents such as acridine orange, acriflavine, proflavine, quinacridine, or 9-aminoacridine), without success. The similarity and ubiquity of the plasmid in all strains of *B. fibrisolvens* tested suggests that the plasmid carries information essential for the survival of the host cell under the growth conditions used. This species shows considerable variability among isolates in its ability to ferment sugars and polysaccharides and to degrade proteins and in its growth requirements (5, 11, 17), and some of this variability may be due to plasmid-determined traits. Experiments designed to determine the structure and function of these plasmids are in progress.

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