

BRIEF COMMUNICATION

Isolation and Initial Characterization of Plasmids in an Acetogenic Ruminal Isolate

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ABSTRACT

Two of nine acetogenic ruminal isolates screened for plasmids were found to contain plasmid DNA. Five plasmids ranging in size from 4.5 to 32 kilobase pairs (kb) were observed in isolate H3HH while a single 35 kb plasmid was observed in isolate H4. The smallest of the plasmids from isolate H3HH, estimated at 4.5 kb, was isolated using gel electrophoresis followed by electroelution. Of the 13 restriction endonucleases tested, this plasmid was cut once by *EcoRV*, *SinI* and *HindIII* and cut twice *BglII*. The physiological functions of the individual plasmids are unknown. However, a plasmid-free derivative (H3HP) of isolate H3HH displayed increased sensitivity to several antibiotics.

Keywords: Acetogens, plasmids, rumen, genetics, bacteria, isolation, characterization

Agric. Food Anal. Bacteriol. 1: 186-192, 2011

INTRODUCTION

Interest in acetogenic bacteria stems from their possible utilization as competitors against methanogens as a hydrogen sink in the ruminal microbial community, for acetate production from inexpensive feedstocks, and their ability to utilize methoxylated phenolics and phenolic acrylates (Drake, 1992; Hespell, 1987; Ragsdale, 1991). Methane production represents a 5 to 15% loss of digestible feed energy to ruminants (Blaxter and Clapperton, 1965). Shifting any portion of the hydrogen used to form methane towards acetate production could result in a concomitant increase in animal productivity.

Although isolation and characterization of plasmids in other ruminal bacteria (e.g., *Prevotella ruminicola*, *Butyrivibrio fibrisolvens*, *Selenomonas ruminantium*) has been reported (Asmundson and Kelly, 1987; Dean *et al.*, 1989; Flint *et al.*, 1988; Flint and Stewart, 1987; Mann *et al.*, 1986; Martin and Dean, 1989; Ricke *et al.*, 1996; Teather, 1982;) the genetic systems of ruminal acetogenic bacteria remain unknown (Forsberg *et al.*, 1986). Analysis of plasmid DNA in acetogens isolated from ruminal contents is of interest because plasmids provide a means towards developing understanding of acetogen physiology and potentially for genetically manipulating acetogens (Dean *et al.*, 1989). This report describes the isolation and initial characterization of plasmid DNA from a ruminal acetogenic bacterium.

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MATERIALS AND METHODS

Organisms and Media

Nine H₂-utilizing, acetate producing isolates (H1 through H9) that had been isolated previously from an H₂ limited continuous culture system (Boccazzi *et al.*, 2011) were grown anaerobically in acetogen medium which contained: (mg/L) K₂HPO₄, 300; KH₂PO₄, 300; (NH₄)₂SO₄, 300; NaCl, 600; MgSO₄ · 7 H₂O, 123; CaCl₂ · H₂O, 80; NH₄Cl, 540; HSeO₃, 0.2; NiCl₂ · 6 H₂O, 20; hemin, 0.01; resazurin, 1; clarified rumen fluid, 5 ml; trace mineral solution (Greening and Leedle, 1989), 10 ml; yeast extract, 500. After the pH of the medium was adjusted to 6.8 with HCl, the medium was prepared using basic anaerobic techniques. The medium was boiled in an appropriately sized reagent bottle while being gassed with oxygen-free CO₂. The bottle was sealed with a rubber stopper, wired in place and autoclaved (121°C, 20 min). After cooling, the flask was transferred into an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) and the following sterile anaerobic reagents were added aseptically (in mg/liter): Na₂CO₃, 4000; cysteine · HCl, 250; Na₂S · 7 H₂O, 250 and vitamin solution (Greening and Leedle, 1989), 10 ml. Twenty ml of media was aseptically dispensed into sterile serum bottles (125 ml) which were then capped with butyl stoppers (Bellco Glass Co., Vineland, NJ).

Cultivation

When H₂-grown cells were desired, a 10% inoculum (from a culture at mid-log phase growing on H₂/CO₂) was aseptically added to serum bottles which were then flushed (for 30 sec) and pressurized to 200 kPa with a 80:20 mixture of H₂/CO₂. These bottles were incubated at least 48 h with vigorous shaking at 37°C. If cells were to be used for plasmid isolation, the medium was supplemented with 0.5% glucose, inoculated (10% v/v), and incubated overnight at 37°C with gentle shaking (100 rpm). When larger amounts of plasmid DNA were needed, the isolates were grown anaerobically in brain heart infusion broth (Difco Laboratories, Detroit, MI) overnight.

Plasmid Isolation

Plasmid DNA was extracted from isolates by the procedure of Sanders and Klaenhammer (1983), with slight modifications: Ten ml of culture were transferred to sterile polypropylene centrifuge tubes (17 x 100 mm, Fisher Scientific Co., Pittsburgh, PA). The cells were harvested by centrifugation (6,000 x g, 7 min, 0°C), washed once with 10 ml of TES buffer (30 mM Tris, pH 8; 5 mM EDTA; 50 mM NaCl), recentrifuged and resuspended in 1 ml of sucrose buffer (50 mM Tris, pH 7.5; 5mM EDTA; 25% (w/v) sucrose). Freshly prepared lysozyme solution (10 mg/ml in 50 mM tris, pH 8) was added to a final concentration of 1 mg/ml followed by incubation (1 h, 0°C). Sphaeroplasts were separated from cell wall debris by centrifugation (4,500 x g, 10 min, 0°C) and resuspended in 0.5 ml of glucose lysis buffer (50 mM Tris; 5 mM EDTA; 50 mM glucose; 3% (w/v) sodium dodecyl sulfate; mixed with 4.3 µl of 10 N NaOH immediately prior to use). After adding proteinase K (final conc. 0.1mg/ml) the pellet was disrupted using a plastic disposable pipette and the lysate incubated (1 h, 62°C). The lysate was slowly cooled to room temperature, 70 µl of 2 M Tris, pH 7 was added, followed by 70 µl of 5 M NaCl. The lysate was transferred to a 1.5 ml microcentrifuge tube along with 0.5 ml of phenol (saturated with 3 % NaCl), emulsified with a vortexer (Genie mixer; Scientific Products, Inc., McGaw Park, IL) for 3 sec and incubated (5 min, RT). To aid in separation of the phases, 0.3 ml of chloroform was mixed in and the emulsion centrifuged (13,000 x g, 5 min, RT). The upper phase (approx 0.6 ml) was removed to a second microcentrifuge tube and 0.6 ml of chloroform:isoamyl alcohol (24:1) added. After a second centrifugation, 0.5 ml of the upper phase was removed to a third tube. DNA was precipitated by adding 2 vol of ice-cold (-20°C) ethanol and incubating (>1 h, -60°C). The precipitated DNA was recovered by centrifugation (13,000 x g, 5 min, RT) and dissolved in 30 µl of sterile distilled water. To reduce RNA interference, 1 µl of DNase free RNase (10 mg/ml) solution was added, followed by incubation (20 min, 37°C).

When larger quantities (greater than 1 mg) of plasmid DNA were required (i.e., for isolation of individual plasmids), a procedure similar to that described by

Anderson and McKay (1983) was used. Three liters of culture were centrifuged (8,000 x g, 7 min, 0°C). Pelleted cells were resuspended in 120 ml of STE buffer (6.7% (w/v) sucrose; 50 mM Tris, pH 8; 1 mM EDTA, pH 8). The cell suspension was divided into four 250-ml polypropylene centrifuge bottles. After adding 7.5 ml of fresh lysozyme solution (20 mg/ml in 50 mM Tris, pH 8) to each bottle, the cells were incubated on ice for 1 h, then at 37°C for 10 min. Next, 3.75 ml of chelating buffer (0.25 M EDTA, pH 8; 50 mM Tris, pH 8) was added to each bottle and the cell suspension swirled for 10 sec. Cell lysis was initiated by adding 2.25 ml of lysing solution (20 % SDS, 50 mM Tris; 20 mM EDTA; pH 8) to each bottle followed by vigorous mixing by hand for 15 sec. Nuclease activity in the cell suspensions was reduced by adding 0.5 ml of proteinase K solution (10 mg/ml in 50 mM Tris, pH 8) to each bottle and incubating for 10 min at 37°C. To complete cell lysis and irreversibly denature chromosomal DNA, 0.6 ml of 10 N NaOH was added slowly while swirling the bottles by hand. The bottles were swirled for an additional 10 min using a gyratory shaker (Model G76; New Brunswick Scientific Co., New Brunswick, NJ).

In order to neutralize the pH of the lysates, 4 ml of 2 M Tris, pH 7 was added to each bottle and swirling continued for 3 additional minutes. After mixing in 5.7 ml of 5 M NaCl, the preparations were centrifuged (9,000 x g, 15 min, 0°C). The supernatant from each bottle was transferred to a second 250-ml centrifuge bottle along with 55 ml of phenol (saturated with 3% NaCl in distilled H₂O). The preparations were swirled for 10 minutes using the gyratory shaker, after which 55 ml of chloroform was added into each bottle. After centrifugation (6,000 x g, 10 min, 0°C), the upper aqueous phase was removed to a clean 250-ml centrifuge bottle with an inverted 25 ml pipette and set aside. Twenty-five ml of TES buffer was added to the phenol:chloroform mixture and vigorously mixed for 30 sec to extract additional DNA from the organic phase. Following centrifugation (6,000 x g, 10 min, 0°C), the upper aqueous phase was removed with an inverted 25 ml pipette and mixed with the aqueous fraction previously set aside. After adding an equal volume of chloroform:isoamyl alcohol (24:1), the two

phases were mixed together, followed by centrifugation (6,000x g, 10 min, 0°C). The upper aqueous layer was removed and DNA was precipitated by adding 2 volumes of ice-cold (-20°C) ethanol and incubation overnight at -20°C. The precipitated DNA was recovered by centrifugation (9,000 x g, 45 min, -5°C) and resuspended in sterile distilled water and pooled. The plasmid DNA preparation was kept at 4°C until used.

Individual plasmids from isolate H3HH were isolated as follows: DNA was electrophoresed through 1 % agarose in TAE buffer (40mM tris-acetate, pH 7.8; 2mM EDTA, pH 7.8) in a horizontal electrophoresis apparatus (Model H5, BRL, Inc., Gaithersburg, MD). The plasmid bands were visualized by incubating the gel in an ethidium bromide solution (0.5 µg/ml) for 10 min and briefly illuminating the gel with ultraviolet light. The gel areas containing the desired plasmid DNA were separated from the rest of the gel with a razor blade. Plasmid DNA was extracted from these gel strips by electroelution as follows: The gel strip was inserted into dialysis tubing (Spectro-por, 12000 to 14000 MWCO, Spectrum Medical Systems, Houston, TX). After removing most of the buffer surrounding the gel slice, the tubing was clamped shut and placed in the electrophoresis unit filled with TAE buffer. The tubing was arranged so that the gel slice was parallel to the electrodes. The plasmid DNA was moved out of the agarose gel slice by applying 5 V/cm (100 V) for 2 h. Following a 1 min polarity reversal of the electric current, the tubing was removed from the unit, unclamped and the gel slice carefully removed. The contents of the dialysis tubing were transferred to a polypropylene centrifuge tube (17x 100 mm, Fisher Scientific Co., Pittsburgh, PA). The inside of the dialysis tubing was rinsed with a small amount of sterile distilled water which was then transferred to the centrifuge tube containing the plasmid DNA. After an initial centrifugation (13,000 x g, 5 min, 0°C) to remove agarose particles, the plasmid DNA was transferred to a microcentrifuge tube, precipitated with ethanol, and pelleted by centrifugation (13,000 x g, 5 min, -5°C). The DNA was dissolved in 50 µl of sterile distilled water and stored at 4°C until used.

Restriction enzyme analysis

Plasmid DNA was digested by restriction endonucleases under the conditions (e.g., salt concentration) specified by the supplier of each enzyme. The plasmid preparations were incubated for 3 h with the appropriate amount of enzymes. When digestions with two different enzymes were performed simultaneously, conditions were adjusted appropriately to optimize activity of both restriction endonucleases. The size of plasmid DNA fragments was estimated by electrophoresis through horizontal 1 % agarose gels using TAE buffer. A 1 kb linear DNA 'ladder' (Gibco BRL, Inc, Gaithersburg, MD) served as the molecular weight marker and was used to estimate the size of plasmid DNA fragments. After electrophoresis, plasmid DNA bands were visualized by soaking the agarose gel in ethidium bromide solution for 20 minutes. The gels were illuminated with a UV transilluminator and photographed with a Polaroid MP-4 Land camera system using type 57 Polaroid film.

Antibiotic sensitivity

The sensitivity of isolates H3HH and H3HP to various antibiotics was determined as follows: 0.1 ml of an overnight culture of isolate H3HH was spread across the surface of 15 cm diameter petri dishes containing brain heart infusion broth (Difco Laboratories, Detroit, MI) solidified with 2% agar. Once the surface had dried, the appropriate antibiotic diffusion disks (Difco Laboratories, Detroit, MI) containing one of the following: novobiocin, vancomycin, kanamycin, chloramphenicol, penicillin G, rifampicin, polymixin B, streptomycin, erythromycin, tetracycline, gentamycin or nalidixic acid were aseptically placed on the agar surface separated by at least at least 3 cm. The plates were incubated anaerobically for 24 h at 37°C and the diameter of the zones of inhibition measured.

Reagents

Restriction endonucleases were obtained from Boeringer Mannheim (Indianapolis, IN) and Prome-

ga, Inc (Madison, WI). Tris, phenol, proteinase K, lysozyme, and ethidium bromide were obtained from Sigma Chemical Co. (St Louis, MO). SeaKem ME agarose was obtained from FMC Corp (Rockland, ME). Gases (H_2 , H_2/CO_2 , N_2 , N_2/CO_2 , CO_2) were obtained from Matheson Gas Products (Joliet, IL). All other reagents were of the highest purity commercially available.

RESULTS AND DISCUSSION

The underlying objective for determining if ruminal acetogenic isolates contain plasmid DNA was to obtain the genetic machinery (i.e. origins of replication) necessary for construction of genetic transfer systems (i.e., shuttle vectors). Although shuttle vectors have been inserted into a non-ruminal acetogen, we felt that use of replicons obtained from plasmids found in acetogens isolated from ruminal contents would enable construction of stable and efficient shuttle vectors between the acetogens isolated from ruminal contents and organisms with well-researched genetic systems such as *E. coli*.

Several procedures, including those described by Birnboim and Doly (1979), Kado and Liu (1981), and Dean *et al.*, (1989) were tested but resulted in poor recovery (both quantitative and qualitative) of acetogen plasmid DNA (data not shown). The methods described herein were adapted from published procedures (Anderson and McKay, 1983; Sanders and Klaenhammer, 1983) designed for use with streptococci and lactococci.

Plasmid DNA was difficult to obtain from these isolates due to high nuclease activity as well as a very tough cell membrane. Deoxyribonuclease activity is not unusual among ruminal bacteria and could pose a barrier to development of gene transfer systems (Flint and Thomson, 1990; Javorsky and Vanat, 1992). However, in isolate H3HH, the deoxyribonuclease activity could be reduced by treating the lysate with proteinase K. Alternatively, when the lysate was heated to 95°C for 10 min deoxyribonuclease activity was reduced, but the reproducibility was variable and at times plasmid DNA could not be visually detected in ethidium bromide (EtBr) stained agarose gels. Incu-

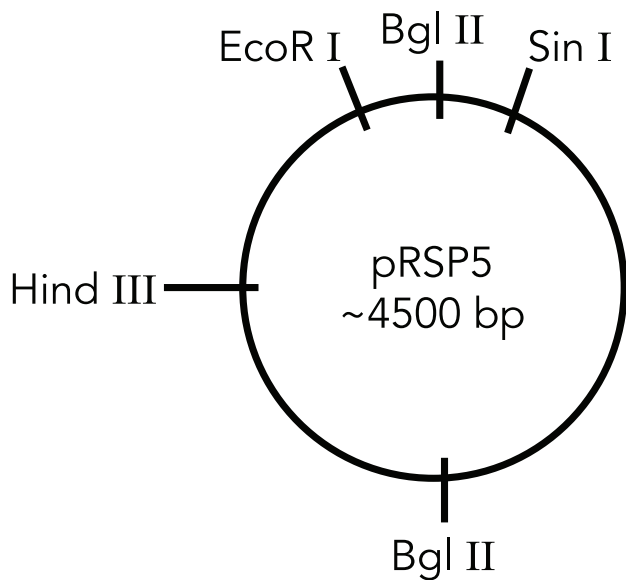


Figure 1. Physical map of pRSP5 obtained from isolate H3HH. Plasmid DNA was electrophoresed through a 0.7% agarose gel and briefly stained with ethidium bromide (0.5 µg/ml). The appropriate band was excised from the gel and the DNA electroeluted out of the agarose. Aliquots of the isolated plasmid DNA were subjected to restriction enzyme digestion and the resulting fragments electrophoresed through a 1% agarose gel, stained with ethidium bromide (0.5 µg/ml) and photographed. The length of the fragments was estimated by comparison with a 1 kb DNA ladder that was also electrophoresed through the gel. The fragments were aligned based on the digestion pattern obtained by simultaneous digestion with two restriction enzymes.

bation of the cell suspension with lysozyme was very important as only minimal lysis was achieved if this step was omitted. A 1 h incubation at 0°C was used for both small- and large-scale preparations but an additional 10 min incubation at 37°C was necessary during the large scale preparation to increase DNA yields. Incubation with mutanolysin, another cell-wall degrading enzyme, was not effective (data not shown).

Isolate H3HH contained plasmid DNA ranging in size from 32 to 4.5 kilobase (kb) (data not shown) while a second isolate (H4) contained a single 35 kb plasmid (data not shown). The presence of plasmid DNA suggests that these isolates may be capable of maintaining foreign DNA, a prerequisite for the exploration of the genetic systems of these organisms. Because of the number of plasmid bands obtained from isolate H3HH (which could be covalently closed circular (CCC), open circular (OC) and linear forms of a few plasmids or CCC form of many plasmids), it was impossible to determine the actual number of plasmids using conventional (unidimensional) agarose gel electrophoresis. However, using the technique of Hintermann *et al.* (1981) where plasmid DNA was subjected to two dimensional agarose gel electrophoresis with ultraviolet light irradiation preceding the second dimension electrophoresis, five plasmid bands were identified in DNA obtained

from isolate H3HH (data not shown). The presence of multiple plasmids in one organism is not unusual and has been reported previously in other bacterial species including: *Escherichia coli* V517 (Macrina *et al.*, 1978), *Lactococcus lactis* (Sanders and Klaenhammer, 1983).

Because of apparent high copy number (estimated by the relative band brightness of EtBr stained gels) and relatively small size, the smallest plasmid of isolate H3HH (named pRSP5) was selected for characterization. A restriction map of the plasmid was constructed by performing single and double digestions with 13 restriction endonucleases (Fig. 1). Although this plasmid was cut by *SinI*, *EcoRV*, *HindIII* and *BglII*, this plasmid did not appear to contain cleavage sites for *PstI*, *Sall*, *EcoRI*, *PvuI*, *PvuII*, *BamHI*, *XhoI*, *SphI*, and *EcoRI*. Analysis of the digested DNA fragments suggested that pRSP5 is a circular molecule of approximately 4.5 kb.

The function of the individual plasmids has not been established because sub-isolates containing a single plasmid band have not been isolated. However, the plasmids are unstable and disappear if isolate H3HH is repeatedly transferred in rich media (such as brain heart infusion). A plasmid-free derivative (H3HP) of isolate H3HH (obtained after more than 10 transfers in brain heart infusion) displayed increased sensitivity to antibiotics, suggesting the presence of

Table 1. Antibiotic sensitivity of isolates H3HH and H3HP

antibiotic	amount / disk	Growth inhibition zone (mm)	
		isolate H3HH	isolate H3HP
Chloramphenicol	30 µg	29 ^a	37
Erythromycin	15 µg	27	36
Gentamycin	10 µg	17	31
Kanamycin	30 µg	10	26
Nalidixic acid	30 µg	8	8
Novobiocin	30 µg	24	43
Penicillin G	2 units	29	39
Polymixin B	300 units	8	23
Rifampicin	5 µg	19	22
Streptomycin	10 µg	7	20
Tetracycline	30 µg	35	48
Vancomycin	30 µg	23	34

^a Overnight broth cultures (0.1 ml) were spread on 100 mm diameter plates containing brain heart infusion medium + 1% agar. Antibiotic disks were placed on the dried surface. Plates were incubated for 48 h inside an anaerobic incubator at 37°C. Values reported are mean (in mm) of two different zones, each in a different plate.

antibiotic resistance genes on these plasmids (Table 1). However, isolate H3HP is capable of growth on H₂/CO₂ at rates similar to isolate H3HH (data not shown), suggesting that these plasmids do not carry genes essential for chemolithoautotrophic growth.

The work described here may open the door for the potential genetic manipulation of acetogens isolated from the rumen. Further research will be needed to determine the genetic information (i.e., origin of replication, promoters, open reading frames, and other components) encoded in pRSP5. The restriction map of pRSP5 will prove helpful for further analysis of this plasmid as well as providing sites for insertion of genes, antibiotic resistance markers or other vectors. Nevertheless, before further work on preparation of shuttle vectors for acetogenic bacteria is performed, a more thorough understanding of the physiology (i.e., determination of control points of the acetyl CoA pathway) and genetics (i.e., analy-

sis of the genes that encode enzymes as well as accessory proteins) of these bacteria is needed. Most, if not all, of the enzymes involved in the acetyl-CoA pathway have been purified to homogeneity and characterized (Ragsdale, 1991). More recently, the complete sequence has become available for certain nonruminant acetogens such as *Moorella thermoacetica* (f. *Clostridium thermoacetum*) which should aid genetic modifications (Pierce et al., 2008).

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