Isolation and Characterization of a Temperate Bacteriophage from the Ruminal Anaerobe *Selenomonas ruminantium*

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A temperate bacteriophage was obtained from an isolate of the ruminal anaerobe *Selenomonas ruminantium*. Clear plaques that became turbid on further incubation occurred on a lawn of host bacteria. Cells picked from a turbid plaque produced healthy liquid cultures, but these often lysed on storage. Mid-log-phase liquid cultures incubated with the bacteriophage lysed and released infectious particles with a titer of up to $3 \times 10^7$ PFU/ml. A laboratory strain of *S. ruminantium*, HD-4, was also sensitive to this bacteriophage, which had an icosahedral head (diameter, 50 nm) and a flexible tail (length, 140 nm). The bacteriophage contained 30 kilobases of linear, double-stranded DNA, and a detailed restriction map was constructed. The lysogenic nature of infection was demonstrated by hybridization of bacteriophage DNA to specific restriction fragments of infected host genomic DNA and by identification of a bacteriophage genomic domain which may participate in integration of the bacteriophage DNA. Infection of *S. ruminantium* in vitro was demonstrated by two different methods of cell transformation with purified bacteriophage DNA.

The rumen of a sheep contains a microbial population density as high as $2 \times 10^{10}$ bacteria per ml consisting of as many as 20 different major species, as well as other minor species, of microorganisms (1, 3). The microflora is largely responsible for the initial breakdown and digestion of feed ingested by the host animal. In view of the high bacterial density, it is not surprising to find bacteriophage also present in the rumen. Indeed, a variety of bacteriophages have been reported (6, 7). However, despite the potential effect of bacteriophage on ruminal bacterial populations, there have been few detailed molecular analyses of any particular bacteriophage, nor have there been any reports of the successful infection of a bacterial culture in vitro by using purified phage DNA. In a report by Hazelwood et al. (G. P. Hazelwood, E. A. Munn, and C. G. Orpin, Abstr. 33rd Annu. Meet. Can. Soc. Microbiol., p. 76, 1983) on the identification of three morphologically similar bacteriophages from isolates of *Selenomonas* spp., two of the bacteriophages were shown to be lysogenic and inducible by mitomycin C. All three were recorded as belonging to morphological type A, with contractile tails, polyhedral heads, and baseplates of various complexities. Little is known, however, of their host range, lytic cycle, or genome size.

In the course of studies on the genetics of ruminal bacteria, we isolated a number of *Selenomonas* strains from crude ruminal samples. Some of these strains were tested for their ability to be infected by bacteriophages from ruminal fluid; infection was observed in one strain. In this report we describe the isolation and characterization of the bacteriophage and its identification as a temperate phase of morphological group B (icosahedral heads and noncontractile, flexible tails). Transformation of host cells in vitro with purified phage DNA and reestablishment of lysogenic cultures are also reported.

**MATERIALS AND METHODS**

**Bacterial growth.** All strains of *Selenomonas ruminantium* used in this study were routinely grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with 500 μg of cysteine per ml and 10 μg of hemin per ml under an atmosphere of oxygen-free 95% carbon dioxide–5% hydrogen on plates in anaerobic jars or in Hungate tubes. For growth on plates, agar was added to 1.2 or 0.6% for top layering. Fresh ruminal isolates of *S. ruminantium* were obtained from sheep, which were maintained on a high-protein diet of lucerne and wheat straw, by spreading crude ruminal samples on M medium (9) and by randomly picking colonies for purification and bacterial classification (1).

**Purification and plating of bacteriophage.** An overnight culture (1 ml) of *S. ruminantium* was mixed with 4 ml of liquid BHI broth containing 0.6% agar at 50°C and immediately plated onto a plate containing BHI broth. No attempt was made to maintain anaerobic conditions at this stage. Bacteriophage were streaked over the lawn of host cells, and the plate was then incubated anaerobically. In some experiments, a suitable dilution of bacteriophage suspension in 10 mM MgSO₄–20 mM Tris (pH 7.4) was added directly to the host cell overlay before the suspension was poured into the plate. For infectivity assays, dilutions of bacteriophage were spotted onto a freshly prepared lawn of host cells, and the plates were inspected for plaque formation after overnight anaerobic incubation.

**Preparation of bacteriophage stocks.** A large bacteriophage plaque was picked in agar, crushed, and incubated overnight in phage dilution buffer. Free bacteriophage were isolated from the supernatant. Bacteriophage were also isolated from the supernatants of freshly lysed cultures that were derived from the inoculation of plaque material into a culture of uninfected bacteria. For the preparation of bacteriophage stocks, an actively growing 100-ml culture of *S. ruminantium* ($A_{600}$, 0.1 to 0.5) was inoculated with 1 ml of bacteriophage suspension containing approximately $10^6$ PFU/ml and incu-
bated until lysis occurred. A few drops of chloroform were added to the lysed culture as a preservative, and the preparation was stored at 4°C for up to several months without any noticeable loss in infectivity. Gradient purification of bacteriophage was achieved by centrifugation of a bacteriophage lysate on a discontinuous density gradient (Nycodenz; Nye-gaard & Co., Diagnostics Division, Oslo, Norway). A sample (0.3 ml) of lysate was layered on top of a gradient containing 1 ml each of 40, 50, and 60% Nycodenz in 10 mM Tris (pH 7.2) and centrifuged in a rotor (Ti60; Beckman Instruments, Inc., Fullerton, Calif.) at 35,000 rpm for 16 h. Fractions (0.4 ml) were collected and analyzed separately.

Preparation of bacteriophage DNA. DNase I (5 mg) was added to 100 ml of a bacteriophage stock prepared as described above and incubated at 37°C for 2 to 3 h. Debris was pelleted by centrifugation at 10,000 × g. NaCl (3 g) and polyethylene glycol (PEG) 6000 (10 g) were added to the decanted supernatant. After the PEG 6000 was dissolved gently, the bacteriophage were left to precipitate on ice for 1 to 2 h. Bacteriophage were pelleted by centrifugation at 20,000 × g for 20 min and then gently dissolved in 1 ml of a solution of 10 mM MgCl₂-20 mM Tris (pH 7.5); 0.1 ml of 0.2 M EDTA and 0.1 ml of 10% sodium dodecyl sulfate was then added, and the bacteriophage suspension was extracted several times with buffer-saturated phenol-chloroform. An equal volume of 3 M ammonium acetate was added, and the DNA was precipitated with 1 volume of isopropanol. After centrifugation, washing, and reprecipitation with ethanol, the DNA was dissolved in 10 mM Tris-0.1 mM EDTA (pH 8.0).

Restriction mapping. Restriction sites were ordered by agarose gel electrophoresis of single and double digests of bacteriophage DNA in the standard manner (5). The positions of some sites were confirmed by probing Southern transfers of restriction digests with subcloned bacteriophage fragments. Subcloning was performed in pUC19 by standard procedures (5). Terminal fragments were identified by limited digestion of the DNA with the exonuclease Bal 31, followed by incubation with an appropriate restriction enzyme and gel electrophoresis. All enzymes were used as recommended by the manufacturer (Pharmacia Fine Chemicals, Piscataway, N.J.). Radioiodinated probes were prepared by the procedure of Feinberg and Vogelstein (2).

Electron microscopy. Bacteriophage from a stock suspension were spotted onto a charged electron microscopy grid and negatively stained with 1% phosphotungstic acid. Grids were examined in an electron microscope (JEM 100CX; JEOL, Ltd.). For sectioning, bacteriophage plaques were picked in a plug of 2% agar and fixed in 2.5% glutaraldehyde in cacodylate buffer (pH 6.8). The agar blocks were then soaked in 2% osmium tetroxide (in cacodylate buffer), followed by dehydration through a series of ethanol washes. Infiltration was done overnight with Spurr epoxy resin, followed by infiltration with fresh epoxy resin and curing for 36 h at 60°C. Blocks were sectioned on an ultramicrotome (LKB Instruments, Inc., Rockville, Md.) to approximately 50 nm. Thin sections were placed on an electron microscope grid, stained with uranyl acetate and then lead acetate, and examined in an electron microscope.

Transformation of S. ruminantium. For transformation by electroporation, a culture of S. ruminantium HD-4 was grown in 50 ml of BHI broth to an A₆₀₀ of 0.4, centrifuged, and suspended in sucrose electroporation buffer as recommended by the manufacturer (Bio-Rad Laboratories, Richmond, Calif.). Bacteriophage DNA (10 μg) was added to the cultures, which were then held on ice for 30 min. Single-pulse electroporation was done at 2,500 V and 25 μF, after which the bacteria were returned to ice for an additional 30 min. By using top agar, the treated bacteria were spread onto BHI broth plates and incubated anaerobically. Plaques were visible overnight. For transformation with PEG, the procedure was based on the method of Smith (8), with the following modifications. Cells were grown anaerobically, without additional magnesium, to an A₆₀₀ of 0.3 to 0.4 corresponding to a cell density of approximately 10⁸ cells per ml before centrifugation and suspension in an equal volume of standard transformation buffer (STB) (8). For some experiments cells were then recentrifuged and resuspended in a 1/10 volume of STB and treated for 10 min with lysozyme (4) at a final concentration of 2 mg/ml. Bacteria were recentrifuged for 1 min and resuspended in the same volume of STB. Bacteriophage DNA (1 to 2 μg) was then added, and the cells were incubated aerobically for 10 min at room temperature before they were treated with PEG 1000 as described by Smith (8). Cells were then centrifuged; suspended in BHI broth plus sorbitol, magnesium chloride, and potassium acetate to the same concentration as that in STB; and plated in top agar onto BHI broth plates. Plaques were visible after overnight anaerobic incubation.

RESULTS

Isolation and characterization of a Selenomonas bacteriophage. Cultures of S. ruminantium were prepared from ruminal samples of a sheep fed on a high-protein diet as described above. Filter-sterilized samples of ruminal fluid were spotted onto lawns of various Selenomonas strains; and one of these strains, designated M-7, developed several phage plaques. The plaques were clear at first, but after several days of incubation they became turbid. Bacteriophage were picked from one plaque and were purified by streaking them onto a fresh lawn of S. ruminantium M-7. A plug of agar from a bacteriophage plaque was crushed in 1 ml of BHI broth medium, and this was used to inoculate 10 ml of a mid-log-phase culture of strain M-7 cells. After 8 h the culture completely lysed in about 30 min. Lysed cultures produced in this way were used as bacteriophage stocks, and titers were determined to be about 3 × 10⁶ PFU/ml. On continued incubation for 1 to 2 days, lysed cultures regrew, eventually attaining a cell density similar to that of an uninfected Selenomonas culture. These cultures could be passaged many times. A lawn of cells from such an infected culture was found to be incapable of supporting the development of bacteriophage plaques and was resistant to further infection. Nevertheless, these cultures always contained some free bacteriophage, and dilutions produced plaques when they were spotted onto lawns of uninfected bacteria. Treatment of infected cultures with 1 μg of mitomycin C per ml failed to induce lysis of cells, but lysis was often observed on cold storage (4°C) of the cultures. Uninfected S. ruminantium cultures did not lyse on storage.

Host specificity of Selenomonas bacteriophage. Drops of serial dilutions of a bacteriophage stock suspension were spotted onto freshly prepared lawns of Escherichia coli HB101, Bacteroides fragilis, Bacteroides ruminicola subsp. brevis B14 and GA-33, S. ruminantium HD-4, and the original host S. ruminantium M-7. After anaerobic incubation, plaques were only observed on lawns of S. ruminantium HD-4 and M-7. At a low dilution, complete clearing of the entire area of the bacteriophage spot was seen. The highest effective dilution was 10⁻⁶, and at this level a few
(<10) plaques occurred on lawns of both *S. ruminantium* strains. This suggests that the bacteriophage infects either strain with equal efficiency.

**Characterization of the *Selenomonas* bacteriophage.** Bacteriophage were concentrated from an infected BHI broth culture of strain HD-4 and prepared for electron microscopy as described above. High-speed centrifugation of a fresh lysate on a discontinuous density gradient (Nycodenz) yielded a light-scattering fraction at a density of 1.22 g/ml. Infectivity assays demonstrated that infectious phage were associated with this fraction, and bacteriophage particles were observed by negative staining and electron microscopy. Electron micrographs of the phage (Fig. 1a) showed a small lambda-like bacteriophage particle with an icosahedral head approximately 50 nm in diameter and 60 nm in length. The tail appeared to be flexible and was approximately 140 nm long. There was some evidence of a baseplate or filamentous structures associated with the tail, but this seemed very fragile and was often lost during phage preparation and spreading. Thin sections of bacteriophage plaques in 2% agar medium were also examined under an electron microscope. Bacteriophage particles were seen within host cells, some of

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FIG. 1. Electron micrographs of *S. ruminantium* bacteriophage. (a) Bacteriophage from a lysed culture were negatively stained with phosphotungstic acid and examined by electron microscopy. Magnification, ×100,000. (b) A bacteriophage plaque in 2% agar was picked from a plate, fixed, and imbedded as described in the text. The plaque was sectioned and examined by electron microscopy. Magnification, ×40,000.
which were in the process of assembly (Fig. 1b); newly released phage particles were also seen. Additional characterization of the bacteriophage was by molecular analysis of its genomic DNA.

**Bacteriophage DNA analysis.** DNA was isolated from bacteriophage particles and analyzed by restriction mapping as described above. The bacteriophage genome was a 30-kilobase (kb), double-stranded DNA molecule and was shown to be linear by progressive enzymatic digestion from the ends with Bal 31 exonuclease, followed by restriction enzyme analysis. Terminal restriction fragments could be assigned because these were degraded first, and then internal fragments were degraded. The ends of the bacteriophage genome are probably cohesive because, on long incubations with restriction enzymes, the two terminal fragments assigned above were frequently lost, with the appearance of an additional fragment equal in size to the sum of the termini (data not shown). A restriction map of the bacteriophage DNA is shown in Fig. 2.

**Investigation of the lysogenic state of the bacteriophage.** The bacteriophage produced plaques that became turbid with age, and stably infected cultures could be derived either from bacteria picked from the turbid center of a plaque or by continued incubation of a lysed culture. This suggested that the bacteriophage were temperate.

To demonstrate this directly, total genomic DNA was extracted from a stably infected culture and from uninfected bacteria. The two preparations were fractionated by agarose gel electrophoresis, transferred to a nylon membrane (5), and hybridized with radiolabeled bacteriophage DNA. The probe hybridized with large-molecular-size (>30 kb) bacterial DNA from infected cultures and with a 30-kb band, probably representing free bacteriophage DNA. There was no hybridization to DNA from uninfected cells. This result suggests that at least a proportion of the bacteriophage DNA is associated with the host genome. When DNA from infected bacteria was digested with BamHI, gel fractionated, and probed with labeled bacteriophage DNA, the hybridization pattern was similar to that of BamHI-digested bacteriophage DNA, except that one fragment was altered in size, possibly representing the site of genomic integration (data not shown).

To determine whether the bacteriophage integrated into the host genome at a precise site, a more detailed restriction analysis of DNA from infected bacteria compared with that from bacteriophage was carried out. Internal BamHI fragments of bacteriophage DNA were subcloned into an E. coli plasmid vector and used to probe Southern transfers of restriction enzyme digests of DNA from infected cells. One plasmid, pINT5, hybridized with the BamHI restriction fragment (described above). This fragment was altered in size compared with the homologous sequence in free bacteriophage DNA and presumably contained both host and bacteriophage sequences. Since the INT5 sequence was internal to the bacteriophage genome, any association with *Selenomonas* genomic DNA could only come about through integration. The integration site on the phage was further localized by analysis of Clal digests. Plasmid pINT5 hybridized not only to a 6.8-kb Clal fragment from bacteriophage DNA but also to a 3.4-kb fragment in DNA from a lysogenic culture of strain HD-4 (Fig. 3). Thus, the integration site in the bacteriophage DNA must be less than 3.4 kb from the nearest Clal site. This result suggests that the integration site of the phage is located near the subcloned fragment INT5, as shown in Fig. 2.

**Transformation of *S. ruminantium* with purified bacteriophage DNA.** Techniques for in vitro packaging of DNA from this or any other ruminal bacteriophage have not yet been established. Therefore, to demonstrate the infectious nature of the isolated DNA and to develop and optimize procedures for DNA uptake into *S. ruminantium*, direct transformation of *S. ruminantium* HD-4 with bacteriophage DNA was investigated. Transformation was tested by two methods, electroporation and PEG-mediated DNA uptake (8). By both procedures, plaques appeared overnight, developing turbid centers over a period of 1 day. No plaques were seen when cells were exposed to the transformation procedures in the absence of bacteriophage DNA. A comparison of the efficiency of transformation by the two techniques showed that PEG treatment was approximately 10-fold more efficient than electroporation, with a frequency of up to 10 transformants per μg of bacteriophage DNA. Higher levels of transformation (up to 5 × 10⁵/μg of DNA) were obtained by

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**FIG. 2.** Restriction map of bacteriophage DNA. Bacteriophage DNA was digested with various restriction enzymes, and the fragments were analyzed by agarose gel electrophoresis. Kbp, Kilobase pairs.

**FIG. 3.** Integration of bacteriophage DNA into the *Selenomonas* genome. Total DNA was isolated from an infected culture of *S. ruminantium* HD-4 and from purified bacteriophage, and a 10-μg portion of each was digested with Clal. Fragments were separated by agarose gel electrophoresis. DNA was transferred to a nylon membrane by Southern transfer and probed with radiolabeled DNA from the bacteriophage subclone pINT5. Lane 1. Bacteriophage DNA; lane 2, *S. ruminantium* genomic DNA; lane 3, ethidium bromide-stained Clal digest of *S. ruminantium* DNA.
pretreatment of the bacteria with lysozyme, as described above (Fig. 4). However, for these experiments, no attempt was made to rigorously establish optimum conditions for each procedure. The optimization of transformation in *Selenomonas* sp. will be reported separately. In the absence of electroporation or PEG treatment, a low level of transformation was sometimes observed, but this was very variable and occurred at a frequency at least 10-fold lower than that in treated cells. These results demonstrate unequivocally that the isolated bacteriophage DNA contains all the necessary information to establish a lysogenic infection in *S. ruminantium* and indicate that transformation of this species with purified DNA can be achieved by either electroporation or PEG-mediated DNA uptake.

**DISCUSSION**

The data reported here describe the isolation and characterization of a temperate bacteriophage that infects the ruminal anaerobe *S. ruminantium*. The phage was infectious on two hosts, strains M-7 and HD-4, suggesting either that there are no restriction barriers between these two strains or that they are identical. The phage has an icosahedral head and a flexible tail, possibly with a baseplate, and belongs to bacteriophage morphological group B. The genome is made up of a linear, double-stranded 30-kb DNA molecule and integrates into the host genome as part of a lysogenic phase of its life cycle. However, the primary response to infection was lysis, with only a very small proportion of infected cells becoming lysogenic. Through division of these cells, plaques with turbid centers formed. Liquid cultures also became lysogenic, and lysis was induced by cold storage. As yet we do not know the molecular basis for partial lysogeny in this bacteriophage, but it could be due to competition between repressor and activator proteins or a consequence of a low integration rate compared with the rate of bacteriophage replication and cell lysis. Since stable lysogenic cultures could be readily established and maintained in vitro, it is likely that all susceptible host strains in the rumen would be in this state. The original detection of the bacteriophage by plaque formation was probably due to random curing of bacteriophage from some bacteria in culture which, after they were passaged in vitro, produced a microbial population that was no longer resistant to infection and lysis by free bacteriophage. Following reinfection, a small proportion of these cells became lysogenic and the rest lysed. Preliminary evidence suggests that the region of the phage responsible for integration is located near a 3.5-kb *BamHI* fragment subcloned into plasmid pINT5. Further work on mapping and defining the functional regions of this bacteriophage is currently in progress.

There are few detailed reports on ruminal bacteriophage, although Richie et al. (7) have described a range of bacteriophages from a number of different ruminal anaerobes. However, Richie et al. (7) performed a morphological study, and no detailed molecular analysis was reported. Hazelwood et al. (Abstr. Annu. Meet. Can. Soc. Microbiol., 1983) have also reported the presence of two bacteriophages in *S. ruminantium*, but no further characterization of these has been reported. Here we have presented one of the first molecular characterizations of a ruminal bacteriophage. Transformation of uninfected *S. ruminantium* HD-4 cells in vitro with naked bacteriophage DNA provided a direct demonstration of the infectious nature of the isolated DNA. Bacteriophage released from cells transformed in vitro were identical to the original bacteriophage and were capable of reestablishing a lysogenic infection. These data not only clearly demonstrate the nature of the isolated DNA but also represent one of the first reports of in vitro transformation of *Selenomonas* sp. Moreover, bacteriophage DNA is an ideal test system for transformation reactions in these bacteria because endogenous plasmids carrying antibiotic resistance markers are not available and the assay does not require the expression of a foreign gene, such as one encoding antibiotic resistance, nor the compatibility of a foreign gene promoter. The assay developed in this study therefore provides a useful expression step for the development and optimization of a bacterial transformation procedure not only for *Selenomonas* sp. but for other species of ruminal bacteria as well. The small size of this bacteriophage and its temperate nature may also form the basis of a vector system for genetic manipulation of *Selenomonas* sp. Further work with this assay is currently in progress, to optimize transformation in *Selenomonas* sp. and to isolate temperate bacteriophage with specificities for other ruminal bacterial species.

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**LITERATURE CITED**