APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1988, p. 534–539
0099-2240/88/020534-06$02.00/0

Vol. 54, No. 2

Use of a Unique Gene Sequence as a Probe To Enumerate a Strain of *Bacteroides ruminicola* Introduced into the Rumen

GRAEME T. ATTWOOD, ROBIN A. LOCKINGTON, GANG-PING XUE, AND JOHN D. BROOKER*

Department of Animal Sciences, Waite Agricultural Research Institute, Glen Osmond, South Australia 5064

Received 28 September 1987/Accepted 25 November 1987

Cloned fragments of genomic DNA from the ruminal anaerobe *Bacteroides ruminicola* subsp. *brevis* B14 were isolated and used as hybridization probes to identify closely related bacterial species. One DNA fragment unique to strain B14 was tested to determine its sensitivity in detecting homologous sequences among total ruminal microbial DNA. In a DNA titration experiment, the probe was capable of detecting strain B14 sequences in vitro down to 0.1% of the total bacterial DNA present in a hybridization assay. There was no detectable signal for total ruminal bacterial DNA. The specificity of this DNA fragment was exploited to enumerate strain B14 in a fresh mixed suspension of ruminal bacteria in vitro and after inoculation of the strain into the rumen. In vitro strain B14 had a half-life of 9 h. However, following inoculation into the rumen there was a very rapid loss of the strain to below the detectable limit within 3 h. The half-life was less than 30 min. This loss was not due to ruminal dilution or to bacteriophage attack but was possibly the result of a specific bacteriocinlike activity present in the rumen and detectable in fresh ruminal fluid.

The rumen contains a complex microbial flora (2, 6) made up of populations of microorganisms that interact and compete for their survival. The behavior of any particular bacterial species within this ecosystem is therefore the subject of considerable interest, especially when it concerns selection for a specific species as the result of a dietary change (20). Recently proposals to introduce genetically manipulated bacteria into the rumen (5, 18) have also raised the question of microbial competition in this environment.

Extensive studies of the existing microflora in the rumen and changes in the microbial population that follow manipulation of the feed supply have been done (20). However, little is known of the mechanisms of competition in the rumen. Nevertheless, the introduction and retention of ruminal bacteria having the capacity for mimosine metabolism (9) into cattle have clearly demonstrated the potential for modified ruminal fermentation through manipulation of the ruminal microflora.

Despite the importance of microbial competition and survival in ruminal function, it has been extremely difficult to analyze changes in microbial populations because of the imprecision of morphological classification techniques for even the most predominant species of ruminal bacteria in ruminal extracts (13). This is particularly true for the pleomorphic *Bacteroides* species. Moreover, culturing ruminal bacteria for the purpose of determining the representation of a specific species is methodologically difficult, involving a series of metabolic tests, and suffers from the criticism that the selection pressure on any one species may change when a population is transferred from the rumen to a culture dish containing either defined or complex medium. No simple selective or differential medium exists for *Bacteroides* species. Similar problems have been encountered in analyzing colonic anaerobes in human fecal material (15).

The most direct way to overcome these difficulties is to use highly specific DNA probes to distinguish between related and unrelated bacterial species and to quantitate the proportional representation of a specific species in a mixed culture. Such a method has the advantages of not requiring complex and time-consuming metabolic tests and of being able to be performed on crude isolates without prior culturing in vitro. DNA-DNA hybridization techniques have been used previously to identify and enumerate *Bacteroides vulgatus* and *B. thetaiotaomicron* in human feces (14, 15), and 16S ribosomal RNA sequence probes have been used to determine phylogenetic relationships among various bacterial species (8).

In this paper, we report the isolation of a DNA sequence unique to *B. ruminicola* subsp. *brevis* B14, a laboratory strain of a common ruminal anaerobe, and the use of this probe in DNA-DNA hybridization experiments to determine the survival time of this organism in a mixed culture in vitro and when introduced into the rumen. Results from these experiments suggest that this *Bacteroides* strain is destroyed rapidly both in the rumen and, to a lesser extent, in in vitro ruminal cultures. There are reports (4, 17) of the existence of bacteriocins produced by aerobic bacteria, and bacteriocins from the anaerobe *Lactobacillus acidophilus* (12) and from the human colonic species *B. fragilis* (16) have been characterized. It would therefore not be surprising to find similar agents in the rumen. We report here evidence for the existence of a *B. ruminicola* subsp. *brevis*-specific bacteriocin in the rumen and preliminary tests of its activity.

MATERIALS AND METHODS

Bacterial cultures and media. Cultures of *B. ruminicola* subsp. *brevis* B14 and GA-33, *B. ruminicola* subsp. *ruminicola* D28F, *Selenomonas ruminantium* HD-4, and *B. fragilis* were grown anaerobically in brain heart infusion broth (Oxoid Ltd., London, England) supplemented with 0.5 mg of L-cysteine hydrochloride per ml, 0.001% hemin, and 1 μg of rezasurin per ml. *Escherichia coli* was grown in LB medium. Cultures of *B. ruminicola* and *S. ruminantium* were obtained from R. Hespell (U.S. Department of Agriculture, Peoria, Ill.). *B. fragilis* was obtained from D. Guiney (University of

* Corresponding author.

Calif.). In vitro incubation of ruminal bacteria was performed in 100 ml of anaerobic culture tubes by mixing 25 ml of fresh ruminal extract with 50 ml of "artificial saliva" as described by McDonald et al. (11). The tubes were mixed thoroughly and incubated at 39°C for up to 2 days. Gas pressure was automatically released through one-way valves in the tops of the tubes.

Isolation of ruminal bacteria. Ruminal fluid samples, including particulate matter, were obtained from sheep through a ruminal fistula. Fibrous material was broken down to small fragments by homogenization on ice for 1 min in an Ultraturrax. The homogenate was squeezed through cheesecloth (four layers), and the filtrate was centrifuged at 100 × *g* for 1 min. The pellet was discarded, and the supernatant was used as a source of ruminal bacteria.

Isolation and cloning of genomic DNA. *B. ruminicola* subsp. *brevis* B14 genomic DNA was isolated from 100 ml of a late-exponential-phase culture by disrupting the cells in a French press at 10,000 lb/in^2 and then extracting the homogenate exhaustively with phenol-chloroform. High-molecular-weight DNA was precipitated with ethanol and recovered by winding out on a glass rod. Following a further ethanol wash, the DNA was dried and dissolved in TE (10 mM Tris, 0.1 mM EDTA [pH 8.0]), and 20 μg was digested with *Eco*RI. The digested genomic DNA was ligated into the phosphatased *Eco*RI site of the cloning vector pUC-19 (19) and transformed into competent *E. coli* HB101 cells (10). Transformants were selected on ampicillin (50 μg/ml) plates, and clones were picked at random for further analysis.

Screening of the library. Plasmids were prepared by alkaline lysis (10) and oligo labeled (3) with [^{32}P]dCTP. Multiple samples of DNA from various bacterial strains were spotted onto a nylon membrane (10) and, after being baked and prehybridized, were hybridized with individual labeled recombinant plasmids in 50% formamide at 42°C. After overnight incubation, the membrane was sequentially washed in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (10) and 0.5× SSC and then autoradiographed.

Characterization of the clone B-18 gene sequence. To demonstrate that clone B-18 DNA was derived from *B. ruminicola* subsp. *brevis* B14, we characterized the cloned fragment by restriction mapping and by hybridization to total B14 DNA which had been digested and fractionated on a 1% agarose gel. The B-18 insert was 1.9 kilobases (kb) long and hybridized to 3.5-kb *Hin*dIII, 3.3-kb *Bgl*II, and 3.8-kb *Pst*I genomic DNA fragments (Fig. 1). The 1.9-kb insert was radiolabeled and used in hybridization assays.

Sensitivity of the *B. ruminicola* gene probes. To test the detection limit of the isolated gene probes, we mixed various numbers of B14 cells with bacteria isolated from fresh ruminal fluid to yield standards ranging from 100 to 0.01% B14 cells. The total number of cells in each standard varied from 1.7 × 10^{11} to 3.4 × 10^{11}. Standard mixtures were centrifuged, and the pelleted cells were snap frozen, thawed quickly, and prepared for DNA extraction as described above. Precipitated DNA was recovered by centrifugation, washed in 70% ethanol, dried, and dissolved in TE. Samples (1, 5, and 10 μg) of each DNA standard were denatured in alkali and spotted, in duplicate, onto Biotrace nylon membranes. One set of standards was hybridized with the labeled pB-18 DNA probe, and the other was hybridized with the pB-8 DNA probe. To ensure that the specific activity of the pB-18 probe was as high as possible for some experiments, the pB-18 insert was not used directly but was excised from the pUC-19 vector and recloned into a Bluescribe (Vector Cloning Systems, Integrated Sciences, Sydney, Australia) vector. This modification allowed transcription of the sequence with the T7 promoter and [^{32}P]CTP to yield an RNA probe with a specific radioactivity of at least 10^9 cpm/μg of DNA template. Hybridization reactions with either RNA or DNA probes were quantitated by cutting out the autoradiographically visualized spots and counting the radioactivity. Calculations from the standards were based on the demonstration that the strength of the B14 hybridization signal reflected the proportion of B14 cells in the original cell mixture.

Inoculation of *B. ruminicola* subsp. *brevis* B14 into the rumen. A 2-liter saturated culture (3 × 10^9 cells per ml) of B14 cells was centrifuged under a CO_2 atmosphere and suspended in 50 ml of brain heart infusion medium. The cells were immediately inoculated, through a fistula, into the rumen of a sheep which had been maintained on a protein-rich diet of lucerne (500 g/day) plus wheaten straw (200 g/day) and from which a preinoculation sample had been taken. The first postinoculation sample was taken at 30 min. Samples were treated as described above for the standards and hybridized with the RNA probe. The DNA standards were always included on the same filter as these samples; therefore, after the hybridization, a direct comparison with the signals from the standards allowed the percentage of B14 cells in the samples to be determined.

Bacterial cell numbers. Bacterial cell numbers were determined by counting, in duplicate, cells present in 10^3- and 10^4-fold dilutions of samples by using a hemacytometer chamber. Cell counting in ruminal samples was performed by first processing crude ruminal fluid as described above and then counting cells in the supernatant. For the in vitro mixed culture experiment and the in vivo ruminal inoculation experiment, bacterial cell numbers were determined by separately counting cell numbers in ruminal samples and in pure *B. ruminicola* subsp. *brevis* B14 cultures and then mixing the two in appropriate proportions.

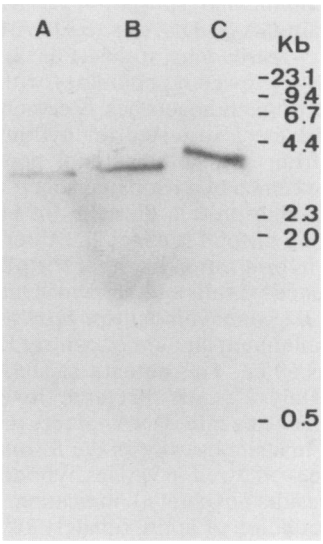

FIG. 1. Characterization of pB-18 DNA. *B. ruminicola* subsp. *brevis* B14 DNA (10 μg) was digested to completion with *Bgl*II (lane A), *Hin*dIII (lane B), and *Pst*I (lane C) and fractionated on a 1% agarose gel. The restriction fragments were transferred to a nylon membrane and probed with radiolabeled pB-18 DNA. Size markers were from *Hin*dIII-digested lambda DNA. Kb, Kilobases.

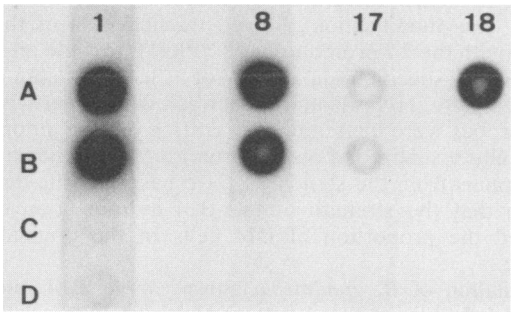

FIG. 2. Identification of *Bacteroides*-specific gene probes. Samples (10 μg) of various bacterial DNAs were multiply spotted onto nylon membranes, separated into strips, and hybridized with different randomly cloned fragments from *B. ruminicola* subsp. *brevis* B14. Rows A to D, Total DNA from *B. ruminicola* subsp. *brevis* B14 and GA-33, *S. ruminantium* HD-4, and *B. fragilis*, respectively. Columns 1, 8, 17, and 18 represent different radiolabeled probes from the strain B14 genomic library.

Bacteriocin assay. Bacteriocin was assayed by the critical dilution method (7), and activity was expressed as the highest dilution above which growth of a standard cell population on agar plates became visible.

RESULTS

Screening of the *B. ruminicola* subsp. *brevis* B14 genomic library. Recombinant plasmids containing chromosomal DNA inserts from *B. ruminicola* subsp. *brevis* B14 were screened in dot blot assays for hybridization to DNA isolated from various *Bacteroides* strains as well as *S. ruminantium* and *E. coli*. A number of recombinant plasmids hybridized to DNA from *B. ruminicola* subsp. *brevis* B14 and GA-33 but not *B. fragilis*, *S. ruminantium* HD-4, or *E. coli*. One plasmid, pB-18, showed specific hybridization to DNA from *B. ruminicola* subsp. *brevis* B14 but did not hybridize to strain GA-33 DNA or to DNA from any other *Bacteroides* or *Selenomonas* strain (Fig. 2). Nonrecombinant plasmid DNA showed no homology with DNA isolated from any of the ruminal anaerobes. Recombinant plasmids pB-8 and pB-18 were also tested for hybridization to total DNA isolated from a mixed bacterial population freshly isolated from the rumen of a fistulated sheep which had been maintained on a high protein diet. In dot blot assays containing up to 10 μg of total ruminal DNA per dot, there was little detectable hybridization of either the pB-8 sequence or the pB-18 sequence (results not shown). Only purified control DNA from *B. ruminicola* subsp. *brevis* B14 reacted in this assay. The minimum amount of control DNA detectable on the blots was 50 ng. This amount of DNA was obtained from approximately 2×10^7 bacteria. In contrast, an *S. ruminantium* HD-4-specific DNA probe (isolated from a genomic library in a similar way as the *B. ruminicola* subsp. *brevis* B14 clone) showed positive hybridization to total ruminal DNA (results not shown), indicating, by reference to standards, a population of approximately 10^8 selenomonads per ml of ruminal sample. These results demonstrate the sensitivity of the assay procedure and show that with DNA probes, bacterial populations in the rumen can be studied directly.

Use of pB-18 to enumerate *B. ruminicola* subsp. *brevis* B14. *B. ruminicola* subsp. *brevis* B14 cells were mixed in increasing proportions with a heterogeneous bacterial population from a fresh ruminal sample. Total DNA was extracted from the mixtures, and 1-, 5-, and 10-μg samples were spotted onto nylon membranes along with appropriate controls. The DNA was hybridized with labeled pB-18 DNA, and after being washed the membranes were autoradiographed. The amount of labeled pB-18 probe which hybridized to the DNA dots increased in proportion to the amount of B14 DNA present in the mixtures (Fig. 3). There was no hybridization signal for ruminal bacterial DNA alone; with 10 μg of DNA from a mixture of ruminal bacteria and strain B14 per spot, a strain B14 hybridization signal could be readily detected when strain B14 cells represented 1% of the total bacterial cell number and, by extrapolation of the data, at least 0.5%. The 10-μg sample of DNA represented approximately 1% of the total DNA extracted from each cell mixture. Sensitivity could be increased to 0.1% when 100 μg of DNA was applied to the nylon membrane (results not shown). In comparing results between subsequent experiments it was important to include internal standards on each hybridization membrane.

Survival of *B. ruminicola* subsp. *brevis* B14 in a mixed culture in vitro and in vivo. The pB-18 probe was used to monitor the survival of strain B14 cells inoculated into mixed ruminal bacterial cultures in vitro and inoculated directly into the rumen of a sheep.

For the in vitro experiment, 25-ml cultures of ruminal bacteria were inoculated with various amounts of strain B14 cells to yield initial proportions of 10 and 50% of the total bacterial cell number. Samples were taken at intervals over 48 h, and total bacterial DNA was prepared as described in Materials and Methods.

For the in vivo experiment, approximately 3×10^{12} or 1×10^{13} cells of strain B14 were introduced, in separate experiments, through a fistula into the rumen of a sheep, yielding

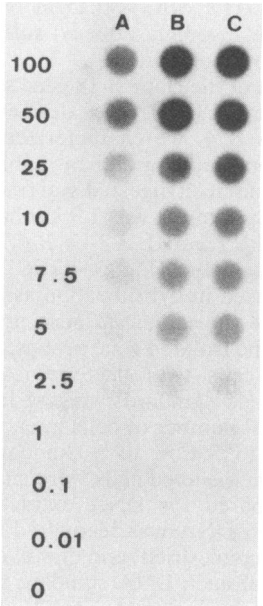

FIG. 3. Sensitivity of *B. ruminicola* subsp. *brevis* B14 detection in a mixture with total ruminal microbial DNA. Strain B14 cells were mixed in various proportions with total ruminal bacteria, and 1 (lane A)-, 5 (lane B)-, and 10 (lane C)-μg samples of DNA from each mixture were spotted onto nylon membranes. The membranes were hybridized to a radiolabeled pB-18 DNA probe. The numbers on the left represent the proportions of strain B14 cells in the mixtures.

an initial inoculum of approximately 3 or 10% strain B14 cells, respectively. Samples of ruminal bacteria were taken after 30 min and then at intervals for the next 2 days. Total bacterial DNA was prepared from these samples as described above.

DNAs from both the in vitro and in vivo experiments were assayed by dot blot hybridization to the pB-18 RNA probe prepared as described in Materials and Methods, and hybridization signals were compared with those obtained for standard DNA mixtures on the same membrane. In the in vitro culture (Fig. 4a), total cell numbers decreased by 50% over 48 h, but strain B14 cell numbers decreased by the same proportion in 9 h. The latter represents a loss of approximately 3×10^8 cells per h as compared with 10^8 cells per h for the total population. By contrast, in vivo the decrease in strain B14 cell numbers was precipitous, such that by 30 min, 25% of the inoculum had disappeared (Fig. 4b). After 3 h, there was no detectable strain B14 DNA in any of the samples, a situation which continued for all subsequent time points. These data represent a loss in vivo of at least 3×10^{12} cells per h. Based on the sensitivity of the assay, these data would represent a population of less than 10^7 strain B14 cells per ml left in the rumen after 3 h of incubation.

Analysis of *B. ruminicola* subsp. *brevis* B14 loss in the rumen. The rapid loss of strain B14 cells from the rumen was faster than could be explained by simple dilution or ruminal turnover and could have been the result of either bacteriophage-mediated lysis or the activity of a specific bacteriocin. To investigate the possibility that a bacteriophage was responsible, we spotted freshly prepared lawns of strain B14 cells with various dilutions of freshly clarified ruminal fluid and incubated them overnight. Zones of inhibition appeared wherever ruminal fluid had been placed. Isolates were taken from these zones, mixed with fresh cultures of strain B14, and replated. If the previous inhibition were due to cell lysis and the release of phage, bacteriophage plaques should then have been seen. Clarified ruminal fluid was also serially diluted, mixed with strain B14 cells, and plated.

There was no evidence of plaque formation upon reinfection. Neither following dilution nor in liquid cultures could reinfection and lysis be demonstrated. Primary growth inhibition mediated by ruminal fluid spotted onto plates was demonstrated repeatedly with fluid taken several times from the same or different sheep, although the critical dilution of bacteriocidal activity varied between different animals. These data suggest the possible presence of a ruminal bacteriocin active against *B. ruminicola* subsp. *brevis* B14.

The specificity of bactericidal activity was tested on lawns of other bacterial strains and species. When diluted (up to fourfold), clarified ruminal fluid was active against *B. ruminicola* subsp. *brevis* GA-33 but was not active against *B. fragilis*, *B. ruminicola* subsp. *ruminicola* D28F, or *E. coli*. There was some activity against *S. ruminantium* HD-4, but only with concentrated ruminal fluid. These data suggest that the rapid loss of strain B14 cells from the rumen may have been due to the presence of a soluble, *B. ruminicola* subsp. *brevis*-specific toxin whose properties resemble those of a bacteriocin (16).

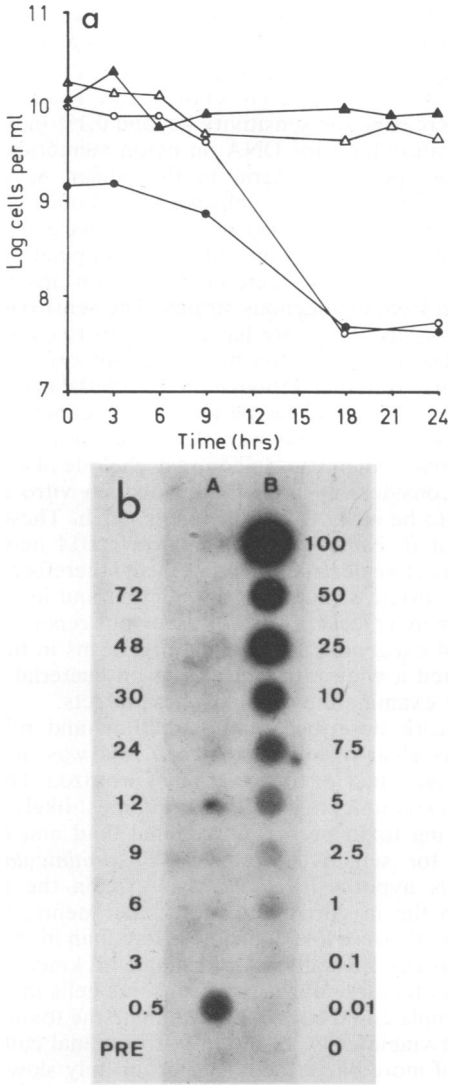

FIG. 4. Survival of *B. ruminicola* subsp. *brevis* B14 in mixed cultures in vitro and when introduced into the rumen. (a) In vitro culture. Strain B14 cells were inoculated into mixed cultures of ruminal bacteria at levels of 10 and 50%. Cultures were incubated, and samples were taken at regular intervals for DNA analysis with the RNA hybridization probe. Parallel samples were used for cell number determination. Data are expressed as equivalent cell numbers based on DNA standard curves. Symbols: ▲, total cell number, 10% inoculum; △, total cell number, 50% inoculum; ●, strain B14 cell number, 10% inoculum; ○, strain B14 cell number, 50% inoculum. (b) In vivo culture. *B. ruminicola* subsp. *brevis* B14 cells were introduced into the rumen at initial inocula of 3 and 10%. Samples were taken at intervals over a 48-h period and analyzed as described above for *B. ruminicola* subsp. *brevis* B14 DNA content. Only results for the 10% inoculum are shown. Lanes: A, 10 μg of sample DNA taken at the times (in hours) shown on the left; B, DNA standards of strain B14-ruminal bacterial mixtures in the proportions (percentages) shown on the right. PRE, Preinoculation.

DISCUSSION

The data presented in this paper demonstrate the use of a specific DNA probe to enumerate a single bacterial strain in a mixed microbial population in vitro and after inoculation into the rumen of a sheep. The sensitivity of the technique is such that at least 50 ng of *B. ruminicola* subsp. *brevis* B14 DNA, equivalent to approximately 2×10^7 cells, can be detected in a single blot. This sensitivity is similar to that reported by Salyers et al. for the enumeration of *B. thetaiotaomicron* (15), although the latter assay procedure involved enzymatic digestion of the DNA and fractionation on an

agarose gel before hybridization. Our results also compare favorably with those obtained with 16S rRNA probes, with which, because of the higher copy number in the genome, a sensitivity of 0.02% has been reported (8). The major limitation to increasing the sensitivity beyond 0.1% in our assay is the saturation limit for DNA on nylon membranes. Enumeration of specific bacteria in the rumen at densities substantially lower than 10^7 cells per ml can only effectively be done by selective culturing and microscopic examination.

One potential problem in ruminal manipulation is the establishment of a new bacterial population against fierce competition from endogenous strains. The sensitivity of the gene probe assay procedure has allowed us to examine this problem. The results of the mixed-culture experiments in vitro demonstrated that although total bacterial cell numbers declined by 50% over the period of the experiment, the introduced strain B14 cells were destroyed at a rate at least threefold greater than this. In contrast, the rate of cell loss in vivo was considerably greater than that in vitro and was calculated to be at least 3×10^{12} cells per h. These results suggest that *B. ruminicola* subsp. *brevis* B14 may be the target of an active destruction process and therefore may not be an appropriate strain for manipulation and introduction into the rumen, at least of sheep. A previous report (1) on the retention of exogenous nonruminal organisms in the rumen demonstrated a wide range of effects on bacterial viability but did not examine the cause of these effects.

In the work described above, dilution and reinfection experiments clearly showed that cell loss was not due to bacteriophage attack or to the action of protozoa. The loss of strain B14 cell numbers was, therefore, most likely due to a rapidly acting toxin present in ruminal fluid and having a specificity for sensitive strains of *B. ruminicola* subsp. *brevis*. This hypothesis would also explain the apparent anomaly in the in vitro incubation experiments, in which strain B14 cells were lost at a slower rate than in the rumen. Since bacteriocins usually act with single-hit kinetics (4), the inoculation of a large quantity of sensitive cells into a small ruminal sample could effectively eliminate the toxin and still leave some viable cells. In the in vitro ruminal culture, the synthesis of more bacteriocin may occur only slowly.

Alternatively, the toxin could be the product of dying ruminal *Bacteroides* cells. If this were the case, the rate of loss of sensitive cells would vary with *Bacteroides* cell density in the rumen or in cultures. Although bacteriocins have not, to our knowledge, been reported in ruminal fluid, they have been described in and isolated from *L. acidophilus* (12), a common ruminal anaerobe, and from the human colonic anaerobe *B. fragilis* (16). It would not be surprising then to find such a toxin in the rumen, particularly because of the selective advantage that such a toxin could give to its resistant producer strain (17), especially under conditions of nutritional deprivation. The sensitivity of strain B14 to such a toxin could also reflect differences between the microbial populations of American cattle (the original host of *B. ruminicola* subsp. *brevis* B14) and Australian sheep.

Preliminary characterization experiments showed that the bacteriocinlike activity appears to be at least subspecies specific, showing selective killing activity against *B. ruminicola* subsp. *brevis* strains but not against other *Bacteroides* strains. There was a slight activity against *S. ruminantium* HD-4, but only with undiluted ruminal fluid. Whether this result reflects the presence of a different toxic agent in the fluid or some nonspecific effect against this laboratory strain is not known. Further work to purify the toxin and identify its producer is currently in progress.

In summary, the results of this work demonstrate the feasibility of using gene probes to study changes in populations of major species of bacteria in the rumen and underline some of the difficulties which may be encountered when attempting to introduce new bacterial strains. On the other hand, a *B. ruminicola* subsp. *brevis*-specific bacteriocin may prove to be an advantage in gene transfer experiments if the toxin were to be plasmid encoded. Since producer strains are usually resistant to bacteriocin attack, if *B. ruminicola* subsp. *brevis* B14 cells were transformed with a bacteriocin-producing plasmid, expression would provide a natural positive selection system in the rumen. A vector constructed from such a plasmid may form the basis of a new genetic transformation system for ruminal bacteria.

ACKNOWLEDGMENTS

We thank D. Jose for excellent technical help throughout this project.

This work was supported in part by the Rural Credits Development Fund of the Reserve Bank of Australia and the Australian Wool Corporation.

LITERATURE CITED

1. **Adams, J. C., P. A. Hartman, and N. L. Jacobsen.** 1966. Longevity of selected exogenous microorganisms in the rumen. Can. J. Microbiol. **12**:363–369.
2. **Bryant, M. P.** 1959. Bacterial species of the rumen. Bacteriol. Rev. **23**:125–153.
3. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137**:226–267.
4. **Hardy, K. G.** 1975. Colicinogeny and related phenomena. Bacteriol. Rev. **39**:464–515.
5. **Hespell, R. B.** 1985. Potential for manipulation of rumen bacteria using recombinant DNA technology, p. 95–100. *In* R. Leng, J. Barker, D. Adams, and K. Hutchinson (ed.), Biotechnology and recombinant DNA technology in the animal production industries. Rev. Rural Sci. vol. 6. **6**:95–100.
6. **Hungate, R. E.** 1966. The rumen and its microbes. Academic Press, Inc., New York.
7. **Jacob, F., L. Siminovitch, and E. Wollman.** 1952. Quantification of bacteriocin levels by critical dilution analysis. Ann. Inst. Pasteur (Paris) **83**:295–315.
8. **Johnson, J. L., and B. Harich.** 1986. Ribosomal RNA homology among species of the genus *Bacteroides*. Int. J. Syst. Bacteriol. **36**:71–79.
9. **Jones, R. J., and R. G. McGarrity.** 1986. Successful transfer of DHP-degrading bacteria from Hawaiian goats to Australian ruminants to overcome the toxicity of *Leucaena*. Aust. Vet. J. **63**:259–262.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **McDonald, P., R. A. Edwards, and J. F. D. Greenhalgh.** 1984. Animal nutrition, 3rd ed., p. 191. Longman, Inc., White Plains, N.Y.
12. **Muriana, P. M., and T. R. Klaenhammer.** 1987. Conjugal transfer of plasmid-encoded determinants for bacteriocin production and immunity in *Lactobacillus acidophilus*. Appl. Environ. Microbiol. **53**:553–560.
13. **Ogimoto, K., and S. Imai.** 1984. Atlas of rumen microbiology, p. 71–124. Japan Scientific Societies Press, Tokyo.
14. **Roberts, M. C., B. Moncla, and G. E. Kenny.** 1987. Chromosomal DNA probes for identification of *Bacteroides* species. J. Gen. Microbiol. **133**:1423–1430.
15. **Salyers, A., S. P. Lynn, and J. F. Gardner.** 1983. Use of randomly cloned DNA fragments for identification of *Bacteroides thetaiotaomicron*. J. Bacteriol. **154**:287–293.

16. **Southern, J. A., W. Katz, and D. R. Woods.** 1984. Purification and properties of a cell-bound bacteriocin from a *Bacteroides fragilis* strain. Antimicrob. Agents Chemother. **25**:253–257.
17. **Tagg, J. G., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40**:722–756.
18. **Teather, R. M.** 1985. Application of gene manipulation to rumen microflora. Can. J. Anim. Sci. **65**:563–574.
19. **Vieira, J. and J. Messing.** 1982. The pUC plasmids. Gene **19**: 259–268.
20. **Warner, A. C.** 1962. Some factors influencing the rumen microbial population. J. Gen. Microbiol. **28**:129–133.