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Isolation and Characterization of a Bacteriocin (Butyrivibriocin AR10) from the Ruminal Anaerobe *Butyrivibrio fibrisolvens* AR10: Evidence in Support of the Widespread Occurrence of Bacteriocin-Like Activity among Ruminal Isolates of *B. fibrisolvens*†

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Forty-nine isolates of *Butyrivibrio fibrisolvens* and a single isolate of *Butyrivibrio crossotus* were screened for the production of inhibitors by a deferred plating procedure. Twenty-five isolates produced factors which, to various degrees, inhibited the growth of the other *Butyrivibrio* isolates. None of the inhibitory activity was due to bacteriophages. The inhibitory products from 18 of the producing strains were sensitive to protease digestion. Differences in the ranges of activity among the *Butyrivibrio* isolates and protease sensitivity profiles suggest that a number of different inhibitory compounds are produced. These findings suggest that the production of bacteriocin-like inhibitors may be a widespread characteristic throughout the genus *Butyrivibrio*. The bacteriocin-like activity from one isolate, *B. fibrisolvens* AR10, was purified and confirmed to reside in a single peptide. Crude bacteriocin extracts were prepared by ammonium sulfate and methanol precipitation of spent culture supernatants, followed by dialysis and high-speed centrifugation. The active component was isolated from the semicrude extract by reverse-phase chromatography. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis confirmed that the peptide was purified to homogeneity, having an estimated molecular mass of approximately 4,000 Da. The N terminus of the peptide was blocked. A cyanogen bromide cleavage fragment of the native peptide yielded a sequence of 20 amino acids [(M)GIQLAPAXYQDIVNXVA AG]. No homology with previously reported bacteriocins was found. Butyrivibriocin AR10 represents the first bacteriocin isolated from a ruminal anaerobe.

The rumen contains a complex microbial community which includes eubacteria, archaea, fungi, and protozoans (21, 22). Working in a concerted fashion, the community converts plant polysaccharides and proteins via anaerobic fermentation to volatile fatty acids and microbial protein, both of which in turn are utilized by the animal. The eubacterial ruminal population consists predominantly of obligate anaerobes (49), of which isolates of *Butyrivibrio fibrisolvens* are generally believed to be a major species (9). Under certain conditions, *Butyrivibrio*-like organisms may form the dominant population within the rumen (9, 49). Related organisms (e.g., *Butyrivibrio crossotus*) have also been isolated from the human intestine (41).

Currently, a single species of *B. fibrisolvens* of ruminal origin is recognized (8). However, the designation as a single species is misleading. Recent findings obtained by 16S rRNA gene sequence analysis (17, 59) have confirmed previous research (8, 37, 38) suggesting that the group is phylogenetically more diverse. Because of their relative abundance within the rumen, *Butyrivibrio* species have been suggested to represent good candidates for genetic manipulations aimed at improving overall rumen function (19, 46, 51).

The inhibitory effects of both rumen fluid and the ruminal environment on nonruminal bacteria are well documented (1, 57). Of greater interest have been reports which suggest that recolonization of the rumen with specific ruminal isolates may also pose some difficulty, in that the introduced strains decline rapidly in numbers (3, 15, 18, 25, 58). The reasons for such declines are not clear. However, it has been suggested that factors such as endogenous bacteriocin production may play a role in affecting colonization (14, 56). An understanding of the factors which allow the rumen to resist colonization will be important, particularly in regard to colonization with recombinant ruminal isolates.

Bacteriocins are a diverse collection of proteinaceous antibacterial agents. The production of these compounds is common throughout gram-positive bacteria (24, 50). To date, the best characterized are those produced by lactic acid bacteria, and several excellent reviews on the occurrence, biological properties, and molecular biology of these compounds are available (5, 31, 32, 44). Many bacteriocins are present in naturally fermented food products and have application for extending shelf life (12, 60) as well as for controlling pathogenic bacteria (13, 30, 36, 48). In general, these compounds tend to be small peptides of 30 to 60 amino acids (24, 50), although examples of larger antimicrobial proteins have been reported (26). The production of bacteriocins is ribosomally mediated and involves a number of posttranslational modifications, including removal of the leader peptide and possible modifications to a number of amino acid residues (24). There is considerable variation in the spectra of activity among these agents.

Bacteriocins and bacteriocin-like activities have been described for a number of strict anaerobes (5, 11, 39, 40, 47) and facultative anaerobes (2, 23, 35, 54) isolated from nonruminal anaerobic sources. In many cases, related organisms have been isolated from the rumen (49). Some evidence suggesting a role for bacteriocins in the overall rumen ecology has been presented (3, 25). Recently, a protease-sensitive factor produced

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by *Ruminococcus albus* was shown to inhibit the growth of *Ruminococcus flavefaciens* in coculture (43). Additional examples of bacteriocin-like activity among facultative anaerobes isolated from the rumen of both sheep and calves have been reported (23, 34). However, facultative anaerobes form only a minor component of the ruminal bacterial community in mature animals, and on this basis it is difficult to assess their impact on the overall rumen ecology.

We have presented evidence which suggests that bacteriocin-like activity is a common characteristic among isolates of *Butyrivibrio* (28). In this report we provide details of the methods and results from the screening of a large collection of *Butyrivibrio* isolates for bacteriocin-like activity. The bacteriocin from one strain (*B. fibrisolvens* AR10) was isolated and partially characterized. This represents the first confirmation of the production of a bacteriocin by a ruminal anaerobe.

MATERIALS AND METHODS

Cultures and growth conditions. All cultures were maintained frozen in glycerol stocks at −20°C until required (53). Growth medium consisted of L10 (10), modified for use in an anaerobic hood (52), containing soluble starch, glucose, and maltose (0.2% wt/vol) as carbon sources. Solid medium consisted of L10 with the addition of 1% (wt/vol) agar (Agar no. 1; Oxoid, Basingstoke, England); overlay agar contained 0.5% (wt/vol) agar. Medium for the growth of nonbutyrivibrio ruminal isolates also contained cellobiose (0.2%, wt/vol). For testing under aerobic conditions, facultative anaerobes and lactic acid bacteria were grown in MRS broth and agar (Oxoid). Anaerobic cultures were grown at 37°C in an atmosphere consisting of H_2-CO_2 (10:90, vol/vol). The sources of cultures are as previously reported (28).

Culture screening. Screening of *Butyrivibrio* spp. for the production of inhibitory end products was carried out by using a deferred-antagonism plating assay (50). In brief, fresh cultures were patched onto square petri dishes (10 cm) containing L10 agar in a grid pattern (nine per plate). Following overnight incubation at 37°C, the plates were removed from the anaerobic chamber, and the growth was washed from the surface of the plate with water and a bent glass rod. The washed plates were sterilized by a brief (5-min) exposure to chloroform vapors, and the chloroform was allowed to dissipate from the plates in a fume hood (5 to 10 min). The plates were placed back into the anaerobic chamber and allowed to rereduce (4 to 5 h) before they were overlaid with 6.0 ml of overlay agar containing 10^6 cells of a fresh overnight culture of the test organism. Overlaid plates were incubated overnight at 37°C, after which they were examined for zones of growth inhibition. In this manner, each of the *Butyrivibrio* isolates was tested against every other isolate.

Zones of growth inhibition were tested for the presence of bacteriophages as follows. A sample of the overlay agar was removed from the zone of clearing with a sterile loop, placed in 1.0 ml of fresh L10 medium, and gently agitated to allow release of phage particles. The preparation was sterilized by passage through a 0.22-μm-pore-size filter, and a 10-μl sample was respotted onto a fresh lawn containing the test strain. Following overnight incubation at 37°C, lawns were examined for evidence of bacteriophage (clearing zone or plaques). All testing was carried out under anaerobic conditions in a glove box.

Isolates found to produce inhibitory activity by the deferred plating technique were tested for the production of inhibitory end products in liquid cultures. Following overnight growth in L10 medium, a 1.0-ml sample of the culture was centrifuged to remove the cells. A 100-μl aliquot was placed on a sterile petri dish and sterilized by exposure to short-wave (180-nm) UV light for 5 min. Inhibitory activity within the UV-sterilized spent culture fluid was assayed by both a drop test and a diffusion well assay (50).

Bacteriocin activity assays. Inhibitory activity in spent culture fluids from *B. fibrisolvens* AR10, and from each step in the purification of butyrivibriocin AR10, were assayed by drop testing 10-μl aliquots of twofold serially diluted sterile samples on top of an agar overlay containing the indicator organism. Activity units were defined as the reciprocal of the highest dilution at which growth inhibition was detectable and were expressed on a per-milliliter basis (critical dilution assay [50]). The overlay agar contained 10^6 cells from a fresh overnight culture of the indicator strain *B. fibrisolvens* OR36. Plates were incubated overnight at 37°C, after which they were examined for evidence of growth inhibition. The dilution buffer (Tris-buffered saline [TBS] [150 mM NaCl, 100 mM Tris, pH 7.5]) used for the titration of butyrivibriocin AR10 contained 0.1% (vol/vol) Tween 20. The inclusion of Tween 20 in the dilution buffer at levels up to 1.0% (vol/vol) had no effect on the growth of the indicator organisms.

Protease sensitivity. The protease sensitivities of the inhibitory end products were determined by two methods. The first method was used to assess the proteolytic sensitivities of the inhibitory end products produced by the various *Butyrivibrio* isolates. A sterile stock solution (10 mg/ml in distilled water [dH_2O]) of each protease (peptidase [porcine intestinal mucosa; Sigma, St. Louis, Mo.], pepsin [Sigma], trypsin [porcine pancreas; Sigma], pronase [protease type XIV; Sigma], and α-chymotrypsin [bovine pancreas; Sigma]) was mixed with 6.0 ml of overlay agar to give a final concentration of 50 μg/ml. The overlay agar was poured over the surface of a deferred assay test plate prepared as described above by washing, $CHCl_3$ sterilization, and rereduction in the anaerobic chamber. The control plate overlay contained no added protease. The plates were incubated at 37°C for 5 h and then overlaid with 6.0 ml of agar containing 10^6 bacteria from a fresh culture of the appropriate indicator. Following overnight incubation, plates were examined for elimination of or reduction in the zones of inhibition by comparison with control plates which contained no added protease.

Determination of the protease sensitivity of butyrivibriocin AR10 was carried out as follows. Ten microliters of a stock solution (10 mg/ml) of each protease was added to a 100-μl aliquot of the purified bacteriocin in TBS plus 0.1% Tween 20 and incubated at 37°C for 2 h, after which activity was assessed by a drop test (50). Controls consisted of protease only (1-in-10 dilution in TBS plus 0.1% Tween 20) spotted directly on an overlay containing the indicator organism.

Isolation of butyrivibriocin AR10. Large-scale cultures (2.0 liters) used for the isolation of inhibitory activity were grown under stationary conditions at 37°C with L10 medium supplemented with additional glucose (2.0%, wt/vol). Resazurin (10) was omitted in the medium used for bacteriocin isolation, as it was found to interfere with subsequent isolation. Following late stationary phase (24 h), Tween 20 was added to the cultures to a level of 0.1% (vol/vol), the cultures were mixed, and the cells were removed by centrifugation (10 min, 10,000 × *g*). Solid ammonium sulfate was added to the supernatant (60 g/100 ml), and the precipitate was collected by centrifugation (30 min, 10,000 × *g*). The resulting pellet was resuspended in 200 ml of dH_2O, and the insoluble materials were removed by centrifugation (30 min, 10,000 × *g*). Previous work had indicated that the majority of inhibitory activity precipitated in the 20 to 40% ammonium sulfate cut. This cut was collected as described above, and the pellet was resuspended in 200 ml of dH_2O. This preparation is referred to as fraction I. Fraction I was mixed with an equal volume of ice-cold methanol and placed at 4°C overnight. Following overnight incubation, the sample was centrifuged (30 min, 10,000 × *g*) to remove precipitated materials. The supernatant was dried under vacuum to remove the methanol, resuspended in 100 ml of dH_2O, and centrifuged (30 min, 18,000 × *g*) to remove insoluble materials. Following centrifugation, the supernatant was washed extensively with dH_2O to remove salts by using an Amicon stirred cell (100-ml volume) fixed with a 3,000-molecular-weight-cutoff ultrafiltration membrane (four wash cycles, with removal of 90% of the volume in each cycle followed by dilution to the original volume). The sample was concentrated to a final volume of 13 ml and subjected to high-speed centrifugation (60 min, 100,000 × *g*) to remove precipitated materials. The supernatant was retained (fraction II). Activity was determined at each point by the critical dilution assay (see above).

The active component was purified from fraction II by fast protein liquid chromatography with reverse-phase chromatography. All steps were carried out at 4°C. Solvents consisted of dH_2O (buffer A) and 2-propanol (buffer B), both of which contained 0.1% trifluoroacetic acid (TFA). A 0.5-ml aliquot of fraction II containing 0.1% TFA was injected onto a Resource RPC 3.0-ml column (Pharmacia, Baie d'Urfé, Canada), washed with two column volumes of buffer A, and eluted from the column with a gradient from 100% buffer A to 100% buffer B at a flow rate of 1.0 ml/min. The elution was monitored at 206 nm. Collected fractions were assayed for inhibitory activity by spot testing, the active fractions were pooled, and the sample was dried in a rotary vacuum centrifuge. The active fraction was redissolved in 0.5 ml of 6 M guanidine-HCl (4), centrifuged (10 min, 16,000 × *g*), filtered through a 0.22-μm-pore-size filter, reinjected onto the column, and eluted as described above. The material eluted as a single peak (fraction III). Samples were stored at 4°C in the elution mixture (80% 2-propanol, 0.1% TFA) until required.

Protein electrophoresis. Samples of butyrivibriocin AR10 (fraction III) were checked for purity by electrophoresis with a Tricine-sodium dodecyl sulfate (SDS)–10% polyacrylamide gel (4) followed by silver staining. The location of bacteriocin activity was determined by overlaying the gel with 0.5% overlay agar containing 10^6 indicator organisms. Prior to being overlaid, the gels were fixed and washed as described by Bhunia et al. (6) and then washed in dH_2O containing 0.1% Tween 20 for 30 min. Following washing, the gel was placed on a glass sheet which was placed in a tray containing moist paper towels, covered with plastic wrap, and allowed to equilibrate under anaerobic conditions for 5 to 8 h prior to overlaying. The overlaid gel was incubated overnight at 37°C and examined for zones of clearing.

Peptide analysis. Samples of purified butyrivibriocin AR10 were dried under vacuum and redissolved in solubilization buffer at 37°C (33). The samples were electrophoresed with Tricine-SDS–10% polyacrylamide gels, after which the peptide was transferred from the gel onto a nylon membrane. Both amino acid analysis and sequence analysis were carried out on samples of the blotted peptide. A CNBr fragment for N-terminal sequence analysis was generated by direct treatment of the blotted peptide sample with CNBr. CNBr cleavage and amino acid and N-terminal analyses of butyrivibriocin AR10 were done in the laboratory of M. Yaguchi, National Research Council, Ottawa, Ontario, Canada.

Testing of nonbutyrivibrio isolates. Nonbutyrivibrio ruminal isolates and nonruminal bacterial isolates were tested as described above by the deferred-antagonism plate assay. Included on each plate was either *B. fibrisolvens* OR36, an isolate which produced no bacteriocin-like activity, or *B. fibrisolvens* OB156, an isolate which produced activity with a very limited spectrum of action. These

controls confirmed that inhibitory activity observed with nonbutyrivibrio test isolates was not the result of nonspecific factors related to the production of inhibitory metabolic end products.

Selected nonbutyrivibrio ruminal isolates were also tested for sensitivity by spot testing with purified butyrivibriocin AR10 (fraction III) dissolved in TBS containing 0.1% (vol/vol) Tween 20. The control consisted of TBS containing 0.1% Tween 20 only. Facultative anaerobes were tested under aerobic conditions with Oxoid MRS broth and agar.

RESULTS

Culture screening. Forty-nine isolates of *B. fibrisolvens* and one isolate of *B. crossotus* were screened for inhibitory end products by the deferred-antagonism plating procedure. Results from this screening are presented in Fig. 1. Twenty-five isolates were found to produce activity which to various degrees inhibited the growth of other isolates. The remaining 25 isolates produced no detectable inhibitory activity under these assay conditions. Each isolate was tested against every other *Butyrivibrio* isolate within the collection. An example of a plate demonstrating deferred growth antagonism in a number of *Butyrivibrio* isolates is shown in the control plate of Fig. 2A. There were marked variations in the sizes of the clearing zones and the degrees of clearing among the strains exhibiting inhibitory activity (28).

Overall, inhibitor production was found throughout all four major 16S rRNA homology groups examined (16, 17), as well as in *B. fibrisolvens* OB235, a strain outside the four major homology groups. Strains within group II showed the highest occurrence of inhibitory activity, the most intense clearing reactions, and the broadest spectrum of activity against the other *Butyrivibrio* isolates (Fig. 1 and 2). These strains were also the least sensitive to inhibitory effects caused by the other producing strains. One isolate from group I, *B. fibrisolvens* AR10, also demonstrated intense clearing reactions throughout the *Butyrivibrio* isolates. Group III and IV isolates demonstrated the lowest incidence of inhibitory activity. Outside the group II isolates, inhibitory activity was generally more specific in terms of the spectrum of activity. None of the clearing zones appeared to be the result of bacteriophages, as replating an extract prepared from overlay agar removed from the center of the clearing zones did not result in either plaques or clearing zones on fresh overlays.

Several of the tested isolates were unusual. Two isolates (*B. fibrisolvens* OR85 and AR27) were not sensitive to inhibition by end products produced by any other strain, nor did they produce any detectable inhibitory activity. *B. fibrisolvens* OR391 produced moderate inhibitory activity but was immune to the inhibitory effects produced by all other isolates. Three isolates (*B. fibrisolvens* OB192, OB194, and ACTF2) were found to be self-inhibitory.

Characteristics of inhibitory activity. With the exception of a single strain (*B. fibrisolvens* AR73), no inhibitory activity was detectable in spent culture fluids from any of the inhibitor-producing strains by either a drop test or a diffusion well assay. Detection of inhibitory activity in spent culture fluids from *B. fibrisolvens* AR73 was a variable characteristic, and inhibition as detected by spot testing was extremely weak.

Protease sensitivities of the inhibitory activity were evaluated by a double-overlay plating method on deferred assay plates. The method was found to be suitable for evaluating the protease sensitivity in most of the strains which produced strong inhibitory activity, providing that an indicator which was not affected by the presence of the protease could be used. Typical results are shown in Fig. 2. Isolates which produced weak levels of inhibitory activity (*B. fibrisolvens* OB156, VV1, UCI12254, OR35, and OB248 and *B. crossotus* ATCC 29175) could not be evaluated by this procedure.

With the exception of *B. fibrisolvens* CF3, all of the tested inhibitory products were sensitive to digestion with pronase but overall quite resistant to digestion with gastric peptidases. Identical sensitivity profiles were found among a number of the inhibitors. *B. fibrisolvens* AR10, AR73, NOR37, and OB236 were sensitive only to pronase or α-chymotrypsin; *B. fibrisolvens* OR76, OR77, OR78, OR84, and OB194 were sensitive only to pronase; and *B. fibrisolvens* X10-C34, OB192, and OR391 were sensitive to pronase, α-chymotrypsin, and trypsin. Strains ATCC 19171, OB251, CF3, GS111, OR79, and OB235 differed in protease sensitivity from the strains mentioned above and from each other.

Low levels of inhibitory activity detectable by spot testing could be extracted from the agar surrounding colonies of a number of high-activity producers (*B. fibrisolvens* AR10, AR73, OR79, OR78, OR77, OR76, and OR85). In the case of *B. fibrisolvens* AR10 and OR79, the inhibitors were tested and found to pass through a membrane with a molecular weight cutoff of 10,000, providing that the extraction was carried out under anaerobic conditions. Exposure of the extracts to the atmosphere resulted in the loss of inhibitory activity.

Production of butyrivibriocin AR10 in liquid culture. A number of additional factors that have been noted to have a positive effect on bacteriocin production in liquid culture in other bacterial species were tested. Inclusion of a solid phase (sterile ground barley or filter paper [28]) or alterations to the initial culture pH, carbon source, or nitrogen source (44) had no effect on induction of inhibitory activity, nor did the addition of spent culture fluids or inocula of different sizes or ages (45) or inclusion of a non-inhibitor-producing sensitive isolate (5).

L10 medium is a semidefined medium and contains no rumen fluid. The medium was designed for the growth and enumeration of a wide variety of ruminal isolates (10). Although all of our strains grew in the medium, we noted a large variation among the *Butyrivibrio* isolates in terms of the quantity of cellular growth. Significant improvements in final culture density resulting from increasing the level of glucose from 0.2 to 2% (wt/vol) yielded detectable levels of protease-sensitive inhibitory activity in filter-sterilized spent culture fluids from both *B. fibrisolvens* AR10 and OR79. Other isolates also demonstrated increased levels of growth under these conditions, but no bacteriocin-like activity could be detected. Further increases in carbohydrate beyond 2% (wt/vol) had no further effect on enhancing production in liquid culture.

Purification of butyrivibriocin AR10. Since several bacteriocins are known to be cell associated (24), we assayed for bound activity on cells of *B. fibrisolvens* AR10 by washing pelleted cells with several nonionic surfactants (Triton X-100, Tween 80, and Tween 20). Triton X-100 was found to be very inhibitory to the growth of *Butyrivibrio* isolates, even at levels less than 0.1% (vol/vol). However, neither Tween 80 nor Tween 20 had an apparent detrimental effect on the indicator strains at levels of up to 1.0% (vol/vol). Significant activity (approximately 50% of the total) was found to be cell associated and extractable with Tween 20 at 0.1% (vol/vol). The inclusion of Tween 20 (0.1%, vol/vol) in spent culture fluid and in the subsequent crude, semicrude, and purified bacteriocin preparations prior to assaying for activity significantly increased activity as determined by critical dilution assay with spot testing.

The inhibitor was isolated from spent culture fluids by a combination of ammonium sulfate precipitation, methanol precipitation, and reverse-phase chromatography (Table 1). The inhibitory activity was eluted from the column as a single

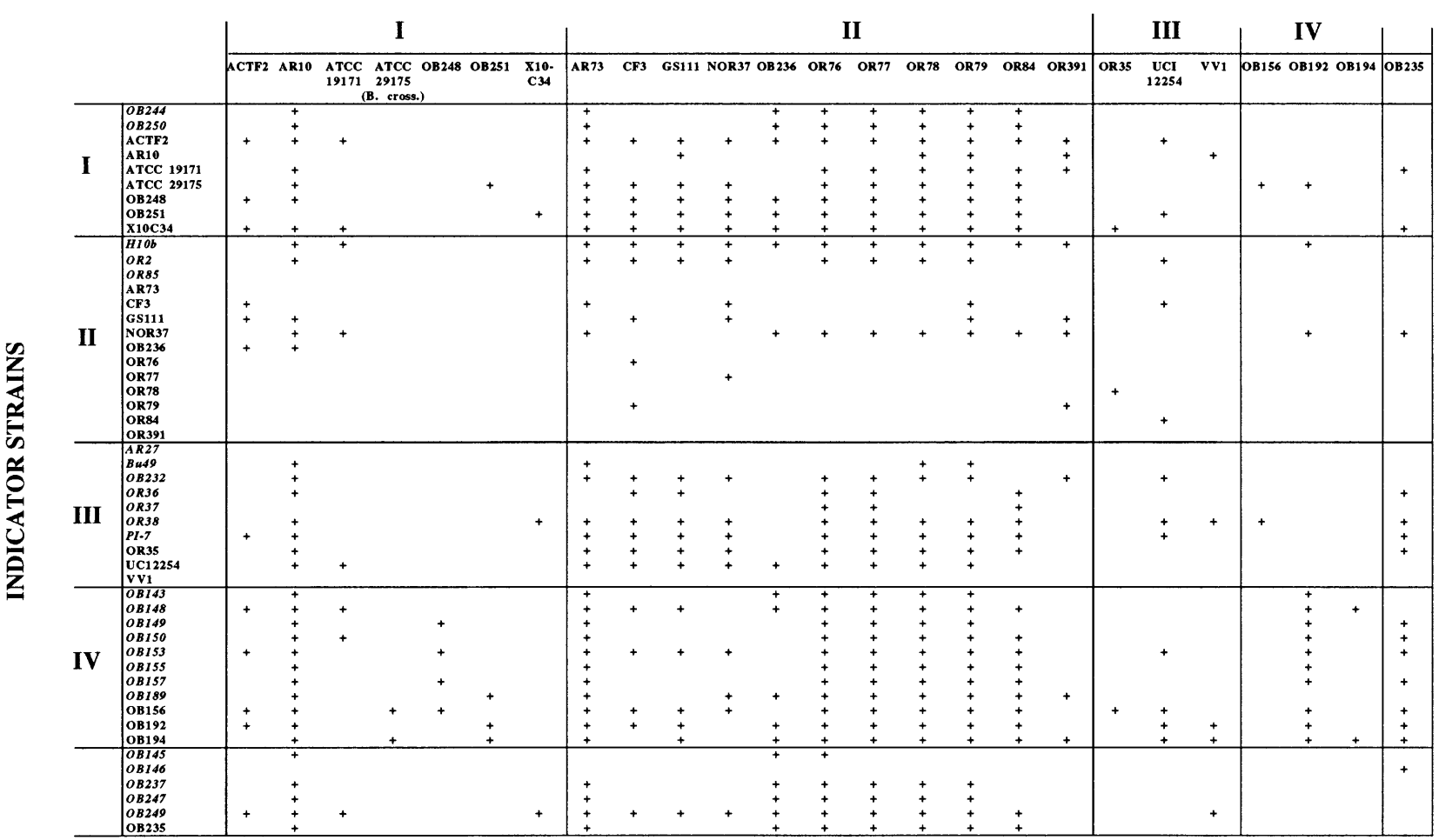

FIG. 1. Summary of large-scale screening of *Butyrivibrio* isolates for inhibitory products by a deferred-antagonism plating method. Strains are grouped on the basis of 16S rRNA sequence. Strain designations of indicator strains that are not producers are italicized.

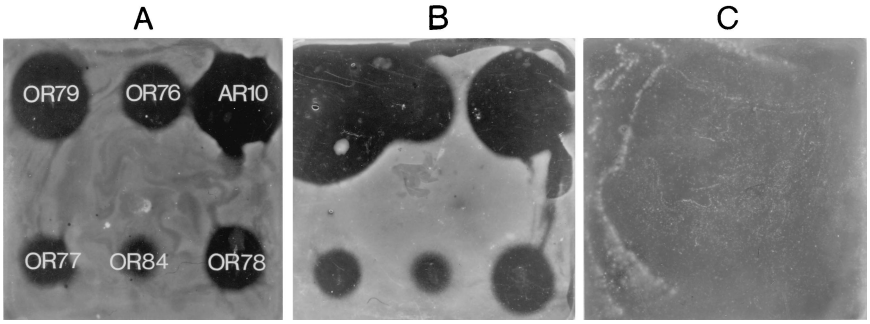

FIG. 2. Double-overlay deferred-antagonism plates demonstrating protease sensitivity of bacteriocin-like activities from an assortment of *Butyrivibrio* isolates. Producers are *B. fibrisolvens* OR79, OR76, and AR10 (top row, left to right) and *B. fibrisolvens* OR77, OR84, and OR78 (bottom row, left to right). (A) control (no added protease); (B) pepsin; (C) pronase. All three plates are overlaid with *B. fibrisolvens* OB156.

peak quite late into the gradient (80% buffer B) (Fig. 3A). Rerunning of the active peak following solubilization in guanidine-HCl confirmed that it ran as a single peak (Fig. 3B).

Tricine-SDS-polyacrylamide gel electrophoresis followed by silver staining indicated that the peak consisted of a single peptide with an estimated molecular mass of 4,000 Da (Fig. 4A). Confirmation that inhibitory activity resulted from the presence of this peptide was obtained by overlaying a Tricine gel containing a sample of the purified peptide with *B. fibrisolvens* OR36 (Fig. 4B). Preparation of the sample by boiling in solubilization buffer did not inhibit activity, whereas treatment of the bacteriocin with pronase completely eliminated inhibitory activity (Fig. 4B, lanes 3 and 4).

Amino acid and N-terminal analysis. Amino acid analysis of the purified bacteriocin (butyrivibriocin AR10), indicated that the peptide had an approximate molecular weight of 3,400. Results of the analysis are presented in Table 2. There were high proportions of both alanine and glycine (nine and five residues, respectively). Neither prior treatment to protect cysteine nor base hydrolysis for the determination of tryptophan was performed.

The N terminus of the native peptide was blocked to Edman degradation. However, the amino acid analysis indicated the presence of a single methionine within the peptide. Treatment of the blotted peptide with CNBr resulted in the generation of an N terminus from which sequence was derived. A 19-amino-acid sequence was obtained [(M)GIQLAPAXYQDIVNXVA AG]. In two cycles (cycles 8 and 15) of the Edman degradation, no major product was identified. No homology with any previously reported bacteriocin sequence was found.

Spectrum of activity among nonbutyrivibrio isolates. Inhibition of non-*Butyrivibrio* bacteria by bacteriocin-like activity of the *Butyrivibrio* isolates was assessed by the deferred-antagonism plating assay. Overall, sensitivities of the non-*Butyrivibrio* strains mirrored those found by testing *Butyrivibrio* isolates. The results are presented in Fig. 5. *Butyrivibrio* isolates in rRNA homology group II and several additional isolates outside this group (*B. fibrisolvens* ATCC 19171, AR10, and OB192) showed broad-spectrum inhibitory activity against other gram-positive ruminal isolates and against a number of food-borne pathogens. Other *Butyrivibrio* isolates also inhibited the growth of the tested strains, although to a lesser extent.

Sensitivities of non-*Butyrivibrio* ruminal isolates to purified butyrivibriocin AR10 were also determined by spot testing. Results from this screening were similar to those found by the deferred assay plating method (results not shown). One isolate (*Streptococcus bovis* ATCC 33317) which did not show any growth inhibition by the deferred-antagonism method demonstrated growth inhibition when tested with the purified bacteriocin. Testing of two facultative anaerobes that were sensitive under anaerobic conditions (*S. bovis* ATCC 33317 and *Lactobacillus ruminis* ATCC 27780) by using aerobic conditions demonstrated no inhibition of growth, confirming a requirement for prior reduction of the bacteriocin to maintain activity.

DISCUSSION

The *Butyrivibrio* species utilized in this study were isolated from diverse geographical and animal sources (28). Each of the isolates conforms with the current definition of *Butyrivibrio* (8). Analysis of 16S rRNA gene sequences has confirmed that these organisms are related (16, 17), although it is likely that they constitute more than two species. Half of the *Butyrivibrio* isolates produced inhibitory activity against the other *Butyrivibrio* isolates when tested with a deferred-antagonism assay. In addition, many of these inhibitory products also appeared to be active against additional ruminal and food bacterial isolates.

Growth inhibition in a deferred assay can result from a number of factors. These include the presence of bacteriocins, bacteriophages, bacteriophage tails, and bacteriolytic enzymes, as well as nonspecific factors relating to the depletion of nutrients or the production of inhibitory metabolic end products

TABLE 1. Purification of butyrivibriocin AR10

Fraction	Vol (ml)	Total A_{280}[a]	Total activity[b]	Sp activity[c]	Relative sp act	Yield (%)
Culture supernatant	1,800	10,026	5.76×10^6	575	1	100
Fraction I	200	1,020	3.20×10^6	3,137	6	55
Fraction II	13	331	2.44×10^6	7,372	13	44
Fraction III	1	1.04	6.24×10^5	6×10^5	1,043	11

[a] Absorbance at 280 nm times total volume (milliliters).
[b] Activity units per milliliter times total volume (milliliters).
[c] Total activity/total absorbance at 280 nm.

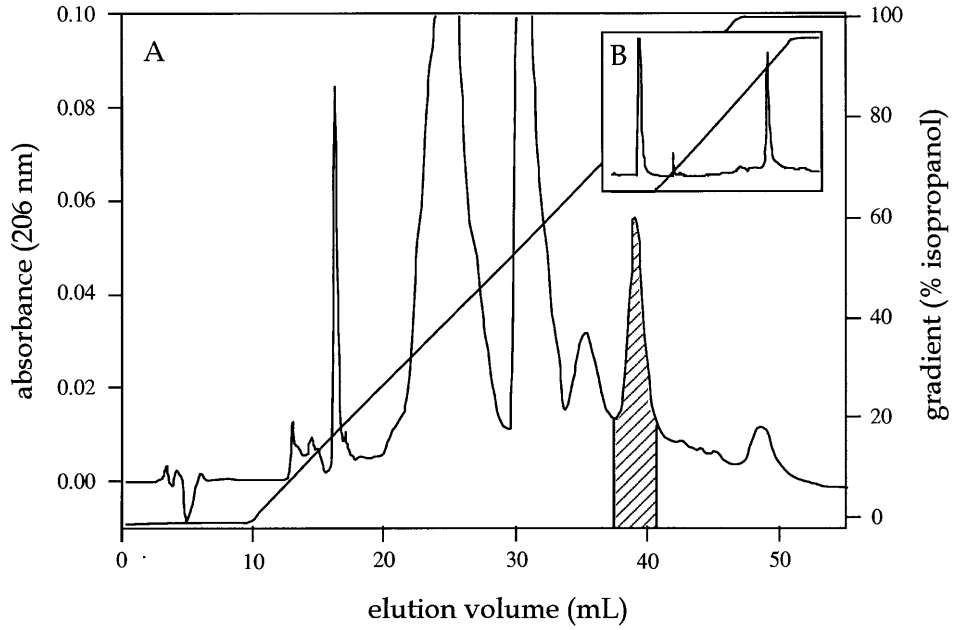

FIG. 3. (A) Reverse-phase chromatographic purification of butyrivibriocin AR10. The peak corresponding to bacteriocin activity is shaded. (B) Rechromatography of the active peak redissolved in 6 M guanidine-HCl.

(24, 44, 50). The inhibitors produced by the *Butyrivibrio* isolates did not appear to be bacteriophages. The majority of the inhibitors produced large inhibition zones, indicating that the inhibitors have low molecular weights, which likely also precludes the possibility that growth inhibition might be the result of bacteriophage tails (7).

Inhibitory effects relating to nonspecific factors (i.e., production of inhibitory metabolic end products) would not be expected to be a factor, since the initial screening process involved the use of a large number of phylogenetically related isolates. In fact, phylogenetically identical isolates could be differentiated on the basis of production or lack of production of inhibitory activity in a deferred plating assay (e.g., *B. fibrisolvens* OR79 and OR85). Differences in terms of activity spectrum throughout the butyrivibrios, sensitivity to proteolytic digestion, and the observation that inhibitor-producing isolates tended to be immune to the growth inhibition effect caused by other producers of inhibitors with a similar spectrum of activity suggest that inhibition is not a result of nonspecific factors. In addition, an inhibitory peptide was successfully isolated from *B. fibrisolvens* AR10. Each of these findings supports the conclusion that the inhibitory activities observed among these isolates are bacteriocin-like. Confirmation that all of these inhibitors are bacteriocins will require both purification and further characterization of these compounds.

Initially, we failed to detect any bacteriocin-like activity in liquid medium with the majority of the tested isolates. In the case of *B. fibrisolvens* AR10 and OR79, improved levels of growth resulting from increased levels of glucose in the medium did yield detectable bacteriocin-like activity in spent culture fluids. Other isolates tested under these conditions also demonstrated increased levels of growth but not the production of the inhibitory activity (results not shown). Induction of

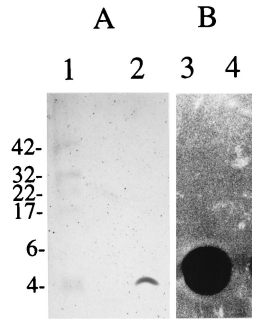

FIG. 4. Tricine-SDS–10% polyacrylamide gel electrophoresis of purified butyrivibriocin AR10 (fraction III). (A) Silver-stained gel. Lane 1, protein standards (with molecular masses in kilodaltons); lane 2, butyrivibriocin AR10, fraction III. (B) Tricine-SDS–10% polyacrylamide gel overlaid with *B. fibrisolvens* OR36 in L10 soft agar demonstrating the zone of growth inhibition. Lane 3, untreated butyrivibriocin AR10; lane 4, pronase-treated butyrivibriocin AR10.

TABLE 2. Amino acid analysis of purified butyrivibriocin AR10[a]

Peak	mol%	Estimated amino acid content
Aspartic acid	6.56	2
Glutamic acid	4.66	2
Serine	5.01	2
Glycine	13.15	5
Threonine	7.13	3
Alanine	26.17	9
Proline	3.47	1
Tyrosine	3.36	1
Valine	5.96	2
Methionine	1.54	1
Isoleucine	9.73	3
Leucine	3.88	1
Phenylalanine	6.25	2
Lysine	2.92	1
Cysteine	ND[b]	ND
Tryptophan	ND	ND

[a] The calculated molecular weight for butyrivibriocin AR10 is 3,418.
[b] ND, not determined.

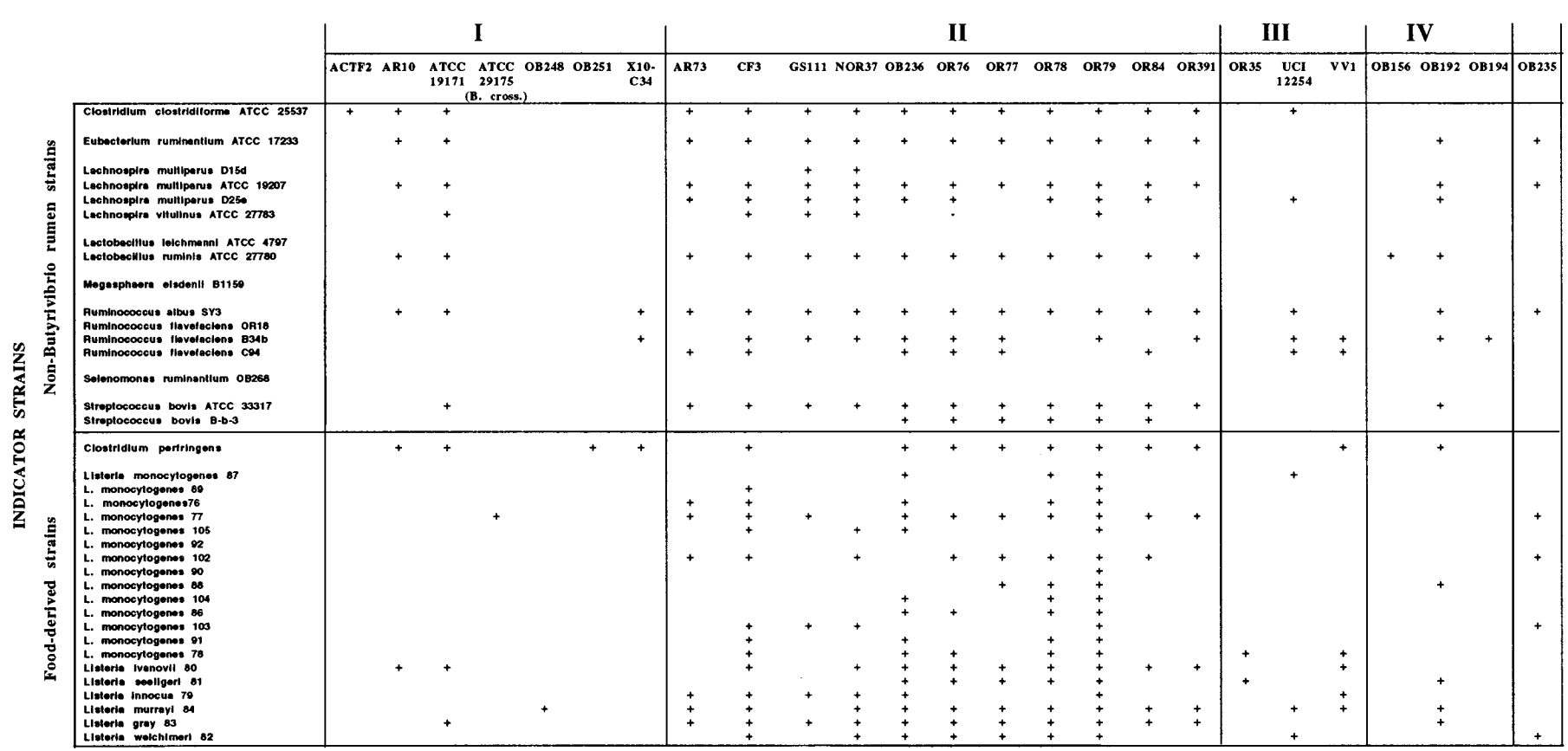

FIG. 5. Sensitivities of ruminal and food isolates to bacteriocin-like activity produced by *Butyrivibrio* isolates as determined by a deferred-antagonism plating method. Strains are grouped on the basis of 16S rRNA sequence.

bacteriocin-like activity or production of detectable quantities of bacteriocin-like activity in liquid culture for these other isolates will likely require further optimization of growth and/or nutritional conditions. Additional factors may also be involved in induction of the bacteriocin-like activities in liquid cultures (5, 45, 50).

The inhibitory activity produced by *B. fibrisolvens* AR10 was the result of a single peptide. Purification of the peptide was carried out by using a combination of ammonium sulfate and methanol precipitation followed by reverse-phase chromatography. Evidence from Tricine-SDS-polyacrylamide gels, total amino acid analysis, and N-terminal amino acid sequencing indicates that the peptide was purified to homogeneity. Based on an identical protease sensitivity profile and spectrum of activity, the peptide isolated from liquid cultures appears to be the same inhibitor detected by the deferred plating assay.

Amino acid analysis of the bacteriocin indicated a very high content of nonpolar amino acid residues. This finding is consistent with the extreme hydrophobicity of the peptide evident from both its elution characteristics with the reverse-phase column and the enhancement in activity which was noted in the presence of nonionic surfactants. Furthermore, activity of the purified bacteriocin in the absence of a surfactant declined significantly during storage, possibly as the result of an association with the surface of the microcentrifuge tubes used for storage. A similar property has recently been noted in several other bacteriocins (27). The hydrophobic nature of the peptide is typical of most bacteriocins (24) and may be a contributing factor in the relatively low levels of recovery during purification (11% yield).

There was some discrepancy (approximately 600 Da) between the molecular mass determination based on amino acid analysis and that from gel electrophoresis. The difference in molecular mass might be due to the presence of several residues of either cysteine or tryptophan. In fact, the oxygen lability of the bacteriocin indicates the presence of at least one reduced cysteine residue. No homology was found between this amino acid sequence and those reported for other bacteriocins. On this basis, this inhibitory peptide represents a new bacteriocin.

The N terminus of the native peptide was blocked to Edman degradation. Other bacteriocins also have blocked N termini (29, 42) or additional modifications to internal residues that block further sequencing (24). Two cycles within the Edman degradation-determined amino acid sequence were blank (positions 9 and 16). In lantibiotics, blank cycles may result from the presence of lanthionine residues (29), but in most proteins, they arise from the breakdown of tryptophan or cysteine residues. Lantibiotics also contain a high proportion of the unsaturated amino acid residues didehydroalanine and didehydrobutyrine, formed from serine and threonine, respectively (24). However, the amino acid analysis indicated the presence of both serine and threonine (two and three residues respectively), which likely precludes the presence of either unsaturated amino acid. We have no evidence which would support the presence of any modified amino acid residues within butyrivibriocin AR10.

One clue to the nature of this bacteriocin, and possibly the additional bacteriocin-like agents from the other *Butyrivibrio* isolates, was the oxygen lability. Treatment of the bacteriocin with β-mercaptoethanol and boiling did not affect the inhibitory activity, suggesting that disulfide bridging is not a requirement for activity. These findings suggest that, like for lactococcin B (55) and possibly sakacin A (20), activity depends on the presence of at least one reduced sulfide group within the molecule. A dependency on anaerobic conditions for activity would carry no penalty within the rumen, where conditions are highly anaerobic.

Our findings suggest that bacteriocin-like activity is common throughout the *Butyrivibrio* group. It is difficult to determine at this point whether this actually reflects normal ruminal populations or is the result of inadvertent selection pressures relating to the manner of isolation. The ecological significance of these inhibitors in terms of the overall function of the rumen has yet to be determined.

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