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An rRNA Approach for Assessing the Role of Obligate Amino Acid-Fermenting Bacteria in Ruminal Amino Acid Deamination

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Ruminal amino acid degradation is a nutritionally wasteful process that produces excess ruminal ammonia. Monensin inhibited the growth of monensin-sensitive, obligate amino acid-fermenting bacteria and decreased the ruminal ammonia concentrations of cattle. 16S rRNA probes indicated that monensin inhibited the growth of *Peptostreptococcus anaerobius* and *Clostridium sticklandii* in the rumen. *Clostridium aminophilum* was monensin sensitive in vitro, but *C. aminophilum* persisted in the rumen after monensin was added to the diet. An in vitro culture system was developed to assess the competition of *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* with predominant ruminal bacteria (PRB). PRB were isolated from a 10^8 dilution of ruminal fluid and maintained as a mixed population with a mixture of carbohydrates. PRB did not hybridize with the probes to *C. aminophilum*, *P. anaerobius*, or *C. sticklandii*. PRB deaminated Trypticase in continuous culture, but the addition of *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* caused a more-than-twofold increase in the steady-state concentration of ammonia. *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* accounted for less than 5% of the total 16S rRNA and microbial protein. Monensin eliminated *P. anaerobius* and *C. sticklandii* from continuous cultures, but it could not inhibit *C. aminophilum*. The monensin resistance of *C. aminophilum* was a growth rate-dependent, inoculum size-independent phenomenon that could not be maintained in batch culture. On the basis of these results, we concluded that the feed additive monensin cannot entirely counteract the wasteful amino acid deamination of obligate amino acid-fermenting ruminal bacteria.

Amino acid deamination in the rumen is a nutritionally wasteful process that often produces more ammonia than the bacteria present can utilize (1). Excess ammonia is absorbed by the animal and converted to urinary urea. Ruminant nitrogen excretion is a major source of environmental pollution, and as much as 50% of the feed nitrogen can be excreted (20). Because ammonia can be oxidized by nitrifying bacteria, nitrite and nitrate accumulate in groundwater (19).

In the early 1960s, Bladen et al. (2) examined the capacity of various ruminal bacteria to deaminate protein hydrolysate and noted that most strains produced little ammonia. On the basis of the bacterial activities and numbers in the rumen, Bladen et al. (2) concluded that *Prevotella* (*Bacteroides*) *ruminicola* was probably the most important ammonia-producing bacterium. Later work, however, indicated that this species could not account for all of the ammonia accumulation in vivo (23).

Ruminal bacteria have traditionally been isolated in media containing carbohydrates and either ammonia or low concentrations of protein hydrolysate as a nitrogen source (3). Hungate (13) noted that he had "encountered" rumen bacteria "able to digest casein and requiring no carbohydrate," but he never isolated these bacteria. In the late 1980s, enrichment cultures containing a high concentration of protein hydrolysate yielded three ruminal bacteria with very high specific activities of ammonia production, and these bacteria could not utilize carbohydrates as an energy source (5, 28). 16S rRNA sequence analyses indicated that these obligate amino acid-fermenting, monensin-sensitive bacteria were *Peptostreptococcus anaerobius*, *Clostridium sticklandii*, and a new species designated *Clostridium aminophilum* (21).

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Most-probable-number estimates indicated that these monensin-sensitive, obligate amino acid-fermenting bacteria accounted for less than 10% of the total ruminal bacterial count (35). Because the feed additive monensin decreased ammonia accumulation in the rumen by 50%, it appeared that obligate amino acid-fermenting bacteria were deaminating a large fraction of the ruminally degradable protein. These estimates were confounded by the fact that many ruminal bacteria cannot be cultured in the laboratory and the observation that obligate amino acid-fermenting bacteria are not the only monensin-sensitive bacteria in the rumen (27). Because 16S rRNA can be used to differentiate bacteria without in vitro culturing (29), we used 16S rRNA probes to assess the contribution of obligate amino acid-fermenting bacteria in vitro and in vivo.

MATERIALS AND METHODS

Basal medium. Ruminal bacteria were grown anaerobically at 39°C in a basal medium containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of $(NH_4)_2SO_4$, 268 mg of NH_4Cl_2, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 4 g of Na_2CO_3, 600 mg of cysteine hydrochloride, 250 mg of Na_2S, vitamins and minerals (11), 3.28 mg of 2-mercaptoethanesulfonic acid, 5 mmol of acetic acid, 2 mmol of DL-2-methylbutyric acid, 2 mmol of isovaleric acid, 2 mmol of isobutyric acid, 2 mmol of valeric acid, Trypticase, and carbohydrates.

PRB. Ruminal contents were obtained from a fistulated cow and squeezed through four layers of cheesecloth. The filtrate was placed in an Erlenmeyer flask at 39°C until the protozoans and feed particles had migrated to the bottom and top of the flask, respectively. A bacterial sample was taken from the center of the flask and transferred to a sterile anaerobic tube. Serial dilutions were made in basal medium containing (per liter) 15 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 1,680 mg of soluble starch, 700 mg of cellobiose, 420 mg of sucrose, 500 mg of xylose, 500 mg of arabinose, and 200 mg of pectin. The 10^8 dilutions produced considerable amounts of succinate, but the addition of a succinate-decarboxylating bacterium (*Selenomonas ruminantium* H18) prevented succinate accumulation in subsequent transfers. The resulting predominant ruminal bacteria (PRB) had cell morphology types that were representative of the cell morphology types found in rumen fluid. Cell morphology was examined

microscopically and remained constant for up to 4 months in a medium containing mixed carbohydrates (see above).

Pure cultures. *Clostridium acetobutylicum* P262 was provided by Francisco Diez, Department of Food Science, Cornell University, Ithaca, N.Y. *Bacteroides uniformis* was provided by Abigail Salyers, Department of Microbiology, University of Illinois, Urbana. *Clostridium paradoxum* ATCC 51510 was provided by Juergen Wiegel, Department of Microbiology, University of Georgia, Athens. *Selenomonas ruminantium* H18 was provided by Herbert Strobel, Department of Animal Science, University of Kentucky, Lexington. All other cultures were obtained from our collection.

Continuous cultures. Continuous cultures were grown anaerobically in basal medium containing 15 g of Trypticase per liter and mixed carbohydrates (see above). The culture vessel volume was 360 ml, and the dilution rate was 0.075 h^{-1}. Monensin (filter sterilized in 95% ethanol) was added to the medium reservoir as needed, and samples were obtained from the culture vessel after a 98% turnover.

In vivo experiments. Two cows were fed chopped timothy hay (9% crude protein, 41% acid detergent fiber, 65% neutral detergent fiber, and 17% nonstructural carbohydrates) with a rotary feeder every 2 h. Monensin was added to the diet to achieve a dose of 350 mg per day. Mixed ruminal bacteria were taken from five locations in the rumen on 2 successive days for each treatment. Samples were frozen at −20°C, and rRNA was extracted (see below). Measurements were performed in duplicate, and the coefficient of variation was less than 10%. Differences were analyzed by using Student's *t* test (30).

Oligonucleotide probe design. Aligned 16S rRNA nucleic acid sequences were obtained from the Ribosomal Database Project (15), and probe sequences that had at least three or four mismatches compared with their closest phylogenetic relatives were chosen. The probe sequences used for *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* were 5′-CTGCGACTTTTGCATCACATTCAGAT-3′, 5′-GCACCGATAAGACGCGCTCG-3′, and 5′-GGCACCGACCTTTGAC AG-3′, respectively. Computer searches were made of the aligned Ribosomal Database Project (15) 16S rRNA sequences and unaligned 16S rRNA sequences available from GenBank. The probes were hybridized with PRB and a phylogenetically diverse group of ruminal and nonruminal bacteria. Bacteria which are predominant in the rumen but for which 16S rRNAs were not available were also included to ensure that ruminal diversity was adequately represented. The dissociation temperatures were estimated experimentally to be 48, 52, and 50°C in 1× SSC (0.15 NaCl plus 0.15 M sodium citrate, pH 7.0) for the *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* probes, respectively. All in vitro hybridization experiments were performed at 48°C, as no cross-hybridizations were ever observed with PRB at this temperature. Hybridizations for the in vivo experiments were conducted at the experimental dissociation temperature of each probe.

rRNA extraction and hybridization. Bacterial samples were immediately frozen at −20°C, and rRNA was extracted from cells by bead beating with a Mini-bead beater (Biospec Products, Bartlesville, Okla.) as described by Stahl et al. (29). Microscopic examination showed that 5 min of bead beating resulted in more than 98% cell breakage. The rRNA concentration was estimated spectrophotometrically at 260 nm and was adjusted to 100 ng/μl. rRNA was denatured by adding 3 volumes of 2% glutaraldehyde and incubating the preparation at 22°C for 10 min. The rRNA sample was diluted with a solution containing polyadenylic acid (1 μg/ml) and bromophenol blue (0.0002%) and applied to nylon membranes (Boehringer Mannheim, Indianapolis, Ind.) in a slot blot apparatus (1 μg of rRNA per slot; Schleicher and Schuell, Keene, N.H.). The membranes were air dried and then baked at 120°C for 30 min.

Hybridization solution (25% formamide, 5× SSC, 50 mM Na_2HPO_4, 2% blocking reagent [Boehringer Mannheim], 2% sodium dodecyl sulfate, 0.1% *N*-lauroylsarcosine) was added (200 μl/cm^2) to each membrane, and the samples were prehybridized at room temperature for 2 h. Digoxigenin-labeled probes (10 ng/ml; Genius 7; Boehringer Mannheim) were added to the hybridization solution, and after 16 h of incubation at room temperature, the membranes were washed twice with 1× SSC for 30 min at 48°C. Probe hybridization was detected autoradiographically by using Lumigen PPD (Boehringer Mannheim) and alkaline phosphatase-labeled anti-digoxigenin antibody (Genius 3; Boehringer Mannheim). The relative amount of 16S rRNA was determined with a universal probe (5′-CCTGTGTCGGTT-3′ [29]), and the amounts of *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* 16S rRNAs were estimated from hybridization intensity standard curves. All reagents used for rRNA extraction and hybridization were treated with diethylpyrocarbonate (Sigma Chemical Co., St. Louis, Mo.).

Other analyses. Cells from continuous cultures were centrifuged at 8,850 × *g* for 10 min at 5°C, washed twice, concentrated, and stored at −20°C. Cells used for protein analyses were stored in 0.9% NaCl, and cells used for rRNA extraction were stored in an RNase-free buffer (29). The ammonia in cell-free supernatants was analyzed by the method of Chaney and Marbach (4). The amount of cell protein was measured by the method of Lowry et al. (18) after the cell pellets were boiled for 15 min in 0.2 N NaOH. The amounts of fermentation acids and carbohydrates were determined by high-performance liquid chromatography with a Beckman model 334 liquid chromatograph equipped with a model 156 refractive index detector and a Bio-Rad model HPX-87H organic acid column; the sample size was 20 μl, the eluant was 0.065 M H_2SO_4, the flow rate was 0.5 ml/min, and the column temperature was 50°C.

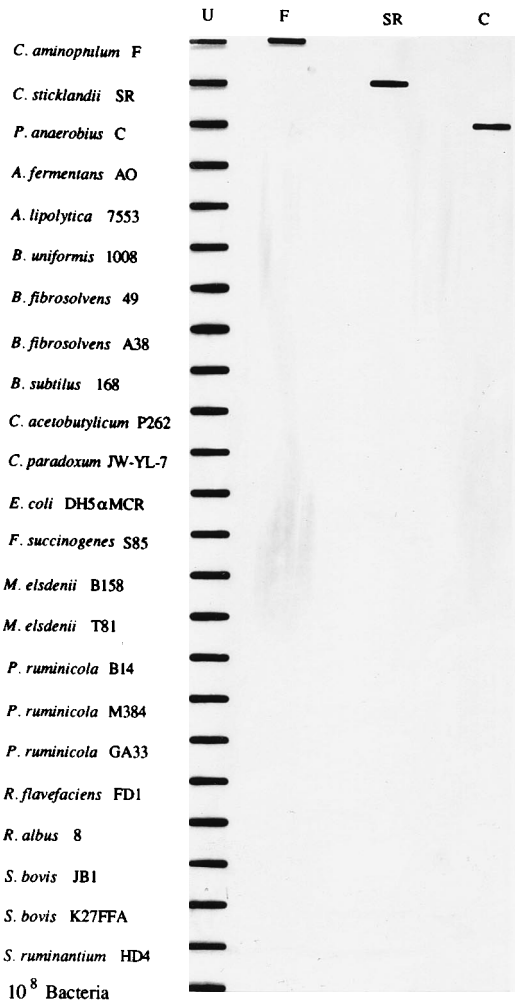

FIG. 1. Hybridization of the universal (lane U), *C. aminophilum* (lane F), *P. anaerobius* (lane C), and *C. sticklandii* (lane SR) 16S rRNA probes with a phylogenetically diverse group of ruminal and nonruminal bacteria. The reference strains used were *Acidaminococcus fermentans* AO (= ATCC 25085), *Anaerovibrio lipolytica* 7553 (= ATCC 33276), *Bacteroides uniformis* 1008, *Butyrivibrio fibrosolvens* 49, *Butyrivibrio fibrosolvens* A38 (= ATCC 27208), *Bacillus subtilus* 168 (= ATCC 6051), *Clostridium acetobutylicum* P262, *Clostridium paradoxum* JW-YL-7 (= ATCC 51510), *Escherichia coli* DH5αMCR, *Fibrobacter succinogenes* S85 (= ATCC 19169), *Megasphaera elsdenii* B158 (= ATCC 17752), *Megasphaera elsdenii* T81 (= ATCC 17753), *Prevotella ruminicola* B_14, *Prevotella ruminicola* M385, *Prevotella ruminicola* GA33 (= ATCC 19188), *Ruminococcus flavifaciens* FD1 (= ATCC 19208), *Ruminococcus albus* 8 (= ATCC 27210), *Streptococcus bovis* JB1, *Streptococcus bovis* K27FFA, and *Selenomonas ruminantium* HD4. 10^8 Bacteria, 10^8 dilution of PRB.

RESULTS

16S rRNA probes. When the 16S rRNA sequences of the obligate amino acid-fermenting ruminal bacteria *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* were compared with sequences obtained from the Ribosomal Database Project (15), it was possible to identify highly specific (hypervariable) regions. Probes for these regions did not cross-react with each other or with 16S rRNAs from individual ruminal or nonruminal bacteria (Fig. 1). Preliminary experiments indicated that the probes hybridized with rRNA from ruminal fluid, and the degree of hybridization was less than 5% (data not shown).

Effect of monensin in vivo. When cows were fed 12 times per day, the rumen achieved a steady state similar to continuous culture. The results of 16S rRNA hybridizations indicated that

TABLE 1. Effect of monensin on volatile fatty acids, ammonia production, and obligate amino acid-fermenting bacteria in the rumen[a]

Characteristic	Result	
	Without monensin	With monensin
Sp act of amino acid fermenters[b]		
C. aminophilum	1.1 ± 0.4	1.4 ± 0.6
P. anaerobius	1.0 ± 0.2	ND[c]
C. sticklandii	0.8 ± 0.2	ND
Ammonia production		
Ammonia (mM)	4.1 ± 0.2	1.8 ± 0.2
SAAP[d] (nmol/mg of protein/min)	30.1 ± 3.1	16.6 ± 1.3
VFA (mM)		
Acetate	44.8 ± 3.5	41.6 ± 1.2
Propionate	11.9 ± 0.6	17.2 ± 0.3
Butyrate	5.2 ± 0.4	5.0 ± 0.2
Valerate	ND	ND
Isobutyrate	ND	ND
Isovalerate	ND	ND
Ratio of acetate/propionate	3.8	2.4
Total	61.9 ± 4.1	63.6 ± 1.5

[a] Cows were fed either no monensin or 350 mg of monensin per day.
[b] All means and standard deviations were the result of multiple extractions and hybridizations.
[c] ND, not detected.
[d] Results are expressed as percentages of the total 16S rRNA.

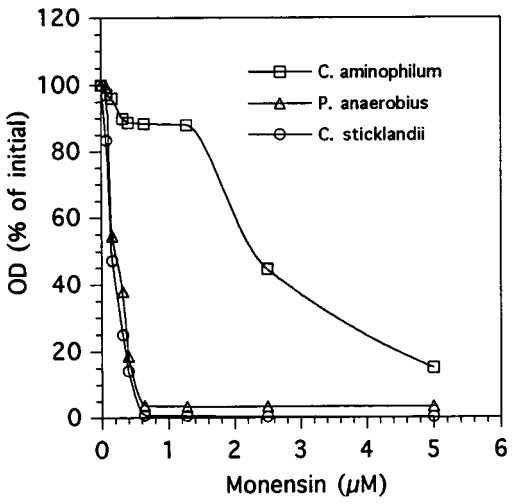

FIG. 2. Effect of increasing levels of monensin on *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* when these organisms were grown in batch cultures containing 15 mg of Trypticase per ml. OD, optical density.

the obligate amino acid-fermenting bacteria were present in the rumen. All three obligate amino acid-fermenting bacteria were present, and each accounted for approximately 1% of the total population (Table 1), and the differences among species were not statistically significant ($P > 0.05$). When monensin (350 mg per day) was added to the diet, the steady-state ammonia concentration declined ($P < 0.05$). Monensin did not cause a significant decrease in volatile fatty acid (VFA) production, but it did cause a significant decrease in the ratio of acetate to propionate. *P. anaerobius* and *C. sticklandii* could not be detected after monensin was added to the diet. *C. aminophilum* was sensitive to monensin in vitro (6), but monensin was not able to inhibit *C. aminophilum* in vivo (Table 1). Monensin even seemed to cause an increase in the *C. aminophilum* population, but animal and day-to-day variation confounded statistical significance ($P > 0.05$).

Effect of monensin on obligate amino acid-fermenting bacteria in batch culture. *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* were all sensitive to monensin in pure culture (Fig. 2), but *P. anaerobius* and *C. sticklandii* were more sensitive than *C. aminophilum*. *P. anaerobius* and *C. sticklandii* could not be grown in batch cultures containing more than 0.5 μM monensin, but *C. aminophilum* could tolerate as much as 1.5 μM monensin. These results, however, did not explain the persistence of *C. aminophilum* in vivo (Table 1). The cows fed monensin had rumen volumes of 70 liters, and the amount of monensin supplied was 350 mg per day. On the basis of a molecular weight of 693, the monensin concentration in vivo could have been as great as 7.2 μM.

Effect of monensin on mixed-culture model system. When ruminal fluid was diluted 10^6 in basal medium containing mixed carbohydrates (3 g/liter) and a high concentration of Trypticase (15 mg/ml), the specific activity of ammonia production was more than 150 nmol/mg of protein per min. Some of the bacteria could utilize peptides and amino acids as energy sources, and hybridization experiments revealed that the obligate amino acid-fermenting bacteria accounted for approximately 50% of the total 16S rRNA. The PRB from 10^8 dilutions had a much lower specific activity of ammonia production (<30 nmol/mg of protein per min). None of these bacteria could utilize amino acids as energy sources, and no hybridization with 16S rRNA probes for obligate amino acid-fermenting bacteria was detected (Fig. 1). The cells of the PRB had various morphologies, and the PRB could be maintained for long periods of time (months) without a loss of morphological diversity.

When the PRB from 10^8 dilutions were grown in continuous culture with mixed carbohydrates (3 g/liter) and ammonia as the only nitrogen source, no residual carbohydrate was detected, and the bacteria utilized approximately 3 mM ammonia. When Trypticase was added to the medium reservoir, the steady-state concentrations of bacterial protein (Fig. 3a), ammonia (Fig. 3b), and VFA (Fig. 3c) increased. Addition of *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* to the PRB resulted in only a small increase in the amount of microbial protein, but the ammonia and VFA concentrations were noticeably higher, particularly at high Trypticase concentrations. The PRB never utilized all of the Trypticase, and the amount of Trypticase that could be recovered as either ammonia or cell protein was as little as 10% (Fig. 4). When *C. aminophilum*, *P. anaerobius*, *C. sticklandii*, and PRB were present, the rate of Trypticase recovery was greater, particularly when the Trypticase concentrations were high. When the Trypticase concentration was less than 1 mg/ml, *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* were not a significant part of the population (Fig. 5), but *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* accounted for as much as 5% of the population when 15 mg of Trypticase per ml was added to the medium reservoir.

Stepwise addition of monensin to continuous cultures receiving 15 mg of Trypticase per ml caused a decline in ammonia and VFA concentrations, but even high concentrations of monensin could not completely reverse the effect of *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* (Fig. 6a). Low concentrations of monensin had little impact on protein synthesis, but addition of monensin eventually caused a large decrease in bacterial protein content (Fig. 6b). Low concentrations of monensin eliminated *P. anaerobius* and *C. sticklandii* from the mixed cultures, but the number of *C. aminophilum* cells was increased by monensin addition (Fig. 7).

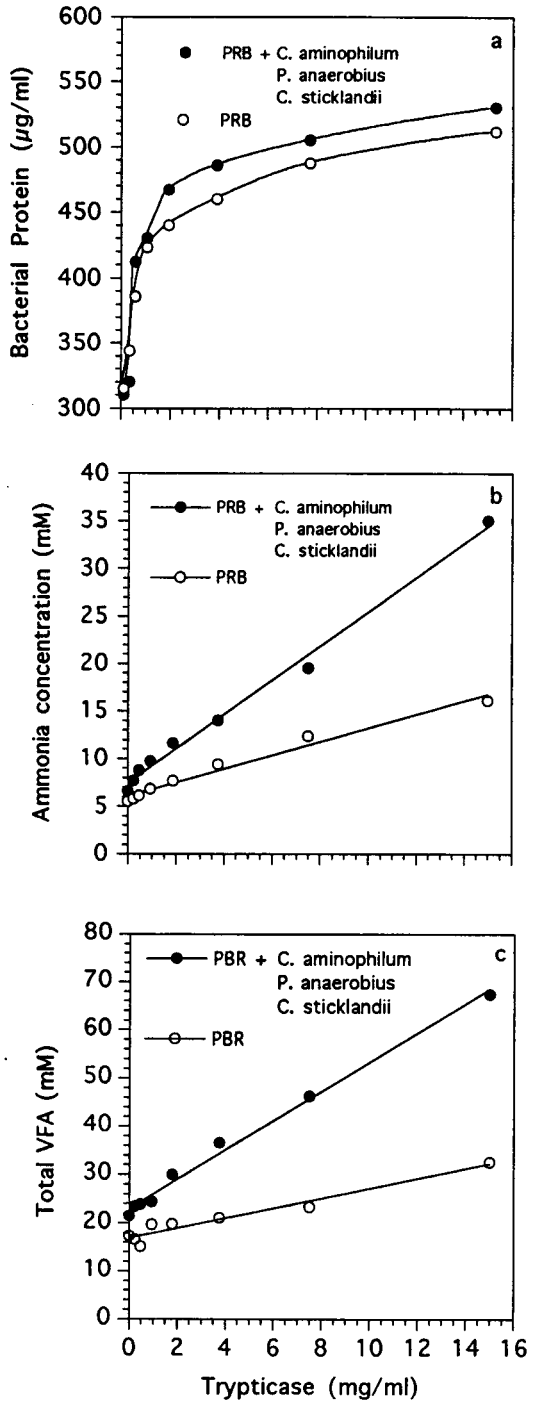

FIG. 3. Effect of Trypticase on the bacterial protein (a), ammonia production (b), and VFA (c) of PRB or PRB plus *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* in continuous cultures (0.075 h^{-1}).

Effect of monensin on *C. aminophilum* in continuous culture. When *C. aminophilum* was grown in continuous culture with 15 mg of Trypticase per ml, stepwise addition of monensin to the medium reservoir had little effect on *C. aminophilum* (less than 10%), and growth was observed at monensin concentrations greater than 5 μM. When the continuous cultures were inoculated into batch cultures, growth was never observed in the presence of 5 μM monensin, even if the size of the inoculum was as great as 50% (data not shown).

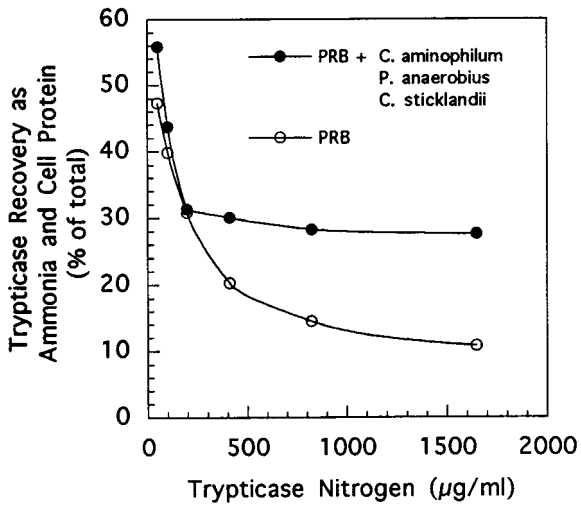

FIG. 4. Trypticase recovery of PRB or PRB plus *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* in continuous culture (0.075 h^{-1}). Trypticase recovery was estimated from the increases in cell protein and ammonia divided by the amount of Trypticase provided.

DISCUSSION

The antibiotic monensin was approved as a ruminant feed additive in 1976, and it dissipates potassium, sodium, and proton gradients across the bacterial cell membrane (22, 24, 27). The outer membrane of gram-negative bacteria excludes monensin from the cell membrane, and gram-negative bacteria are generally more resistant to monensin than gram-positive bacteria (26). Monensin was initially marketed as a methane inhibitor, but its effect on hydrogen production and methane accumulation explained only part of its benefit (27).

The effects of monensin on cellulose digestion are contradictory (32, 33). In the 1980s, Stahl and his colleagues (29) used 16S rRNA probes to assess the impact of monensin on *Fibrobacter succinogenes*, the only monensin-resistant cellulolytic bacterium that has ever been isolated from the rumen (9). It had generally been assumed that *F. succinogenes* would be the dominant cellulolytic bacterium in monensin-fed cattle (28). The results of the 16S rRNA studies of Stahl et al. (29) indicated that *F. succinogenes* persisted in the rumen after monensin was added, but the measurements of these authors were confounded by nearly fivefold day-to-day variations. Because the genus *Fibrobacter* is a very diverse bacterial group (16, 17), the 16S rRNA probes may have been too specific.

Despite the observation that monensin decreased amino acid deamination in vivo (12) and in vitro (31), the amino acid-sparing effect of monensin was largely ignored. The obligate amino acid-fermenting bacteria are gram positive and monensin sensitive, but the amino acid-sparing effect of monensin is complicated by the observation that amino acids are also deaminated by carbohydrate-fermenting ruminal bacteria. When cattle were fed monensin, the most probable number of obligate amino acid-fermenting bacteria declined 10-fold (35), but the values obtained did not give information regarding the relative numbers of each bacterium.

Meaningful studies of bacterial ecology are dependent on representative sampling procedures, quantitative methods of enumeration, and a physiological basis for assessing interactions. In this regard, the obligate amino acid-fermenting rumi-

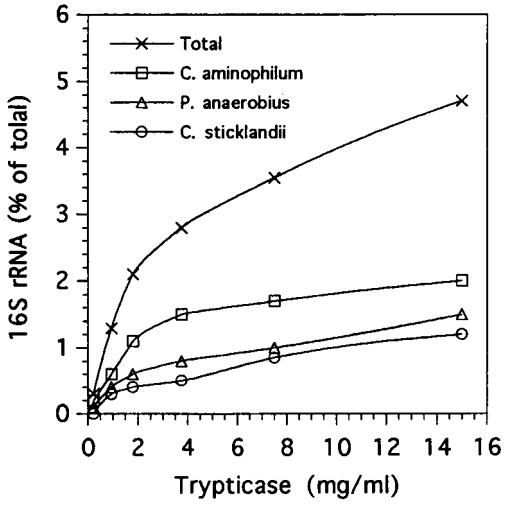

FIG. 5. Effect of increasing levels of Trypticase on the proportion of the total 16S rRNA that hybridizes with *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* probes. A 10^8 dilution of PRB, *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* were grown together in a continuous culture.

nal bacteria were an ideal model. These organisms (i) utilize soluble substrates, (ii) are the only ruminal bacteria isolated so far that grow rapidly on peptides and amino acids, (iii) can be differentiated by their high specific activities of ammonia production, (iv) are monensin sensitive, and (v) have unique 16S rRNA sequences.

Our in vivo studies indicated that monensin decreased ammonia production and that this decrease was correlated with a decline in the amount of 16S rRNA that hybridized with probes for *P. anaerobius* and *C. sticklandii*. The inability of monensin to inhibit *C. aminophilum* was an observation that could not be explained initially. *C. aminophilum* does not have a large a proton motive force across its cell membrane (7), but it has a monensin-sensitive sodium gradient that is needed for amino acid transport and ATP formation (7). Because previous work indicated that monensin could bind to monensin-resistant ruminal bacteria as well as feed particles (10), it was possible that the effective dose of monensin in vivo was lower than the dose predicted solely by intake and ruminal volume.

In vitro experiments can be controlled more carefully than in vivo experiments, but in vitro studies can be easily confounded by *Streptococcus bovis*, an opportunistic bacterium that outgrows other ruminal bacteria when carbohydrates are plentiful (14, 25). The PRB from highly diluted ruminal fluid (fluid diluted 10^8-fold) could be maintained as a diverse population that did not contain *Streptococcus bovis*, *C. aminophilum*, *P. anaerobius*, or *C. sticklandii* and had a low specific activity of ammonia production. On the basis of these results, it was possible to assess the relative roles of PRB and obligate amino acid-fermenting bacteria in vitro.

The in vitro continuous culture experiments indicated that PRB converted some of the Trypticase to ammonia, but ammonia production increased significantly when *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* were added. *C. aminophilum*, *P. anaerobius*, or *C. sticklandii* derives little energy from amino acid fermentation and must ferment approximately 20 amino acids in order to polymerize a single amino acid into protein (5, 6). Given this observation, it is not surprising that addition of *C. aminophilum*, *P. anaerobius*, or *C. sticklandii* had little effect on the steady-state concentration of microbial protein. The obligate amino acid-fermenting bacteria accounted for less than 5% of the total 16S rRNA in continuous culture, and this result was consistent with the observation that *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* are found at low numbers in the rumen but have much higher specific activities of ammonia production than other ruminal bacteria (35).

Previous work indicated that Trypticase contained a pool of peptides that was highly resistant to ruminal degradation (8) and that undegraded Trypticase can pass out of the rumen (34, 36). The PRB never utilized all of the Trypticase, but addition of *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* caused an increase in Trypticase utilization. When monensin was added to the continuous cultures, ammonia production declined 33%. A similar decrease (30 instead of 33%) was observed when cattle were fed a protein-rich ration supplemented with monensin (35), and these results supported the idea that an in vitro mixed-culture system provided a realistic model of monensin-dependent amino acid sparing.

The in vitro continuous culture experiments likewise indicated that monensin did not inhibit *C. aminophilum*. However, *C. aminophilum* was monensin resistant only in low-dilution-rate continuous cultures that mimicked the rumen. Because monensin resistance was not retained by batch cultures, it appeared that monensin resistance was a growth rate-dependent phenomenon. The monensin resistance of *C. aminophi-*

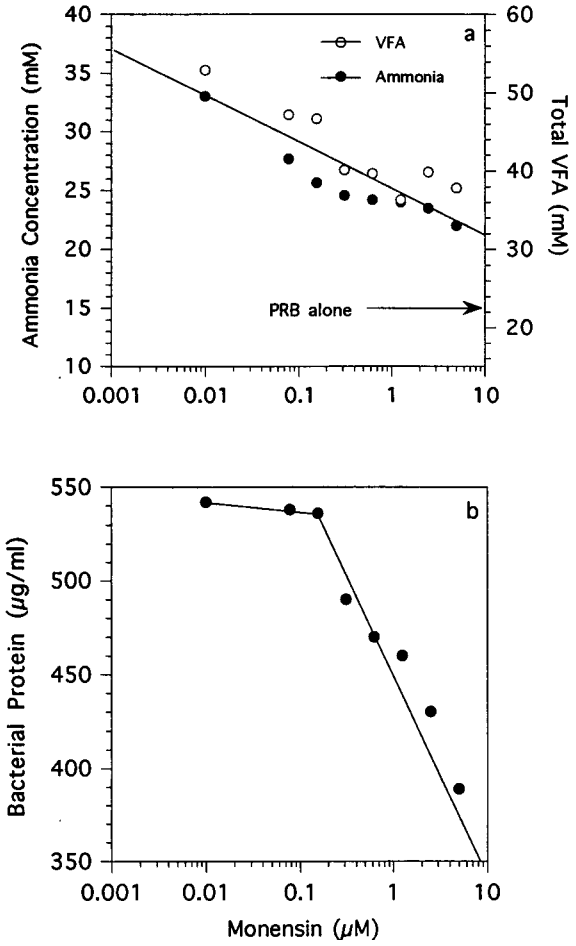

FIG. 6. Effect of increasing levels of monensin on ammonia (a) and protein concentrations (b). *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* were grown together with a 10^8 dilution of PRB in a continuous culture.

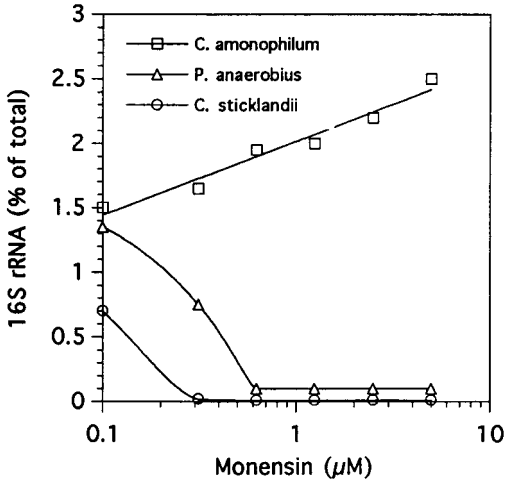

FIG. 7. Effect of supplemental monensin on *C. aminophilum*, *P. anaerobius*, and *C. sticklandii* total 16S rRNAs when these organisms were grown together with a 10^8 dilution of PRB in a continuous culture.

lum reduces the amino acid-sparing effect of monensin. When a bacterial percentage of 1.4% (Table 1), a bacterial protein concentration of 1.1 mg/ml (35), a yield for *C. aminophilum* of 3.3 mg of protein per mmol of amino acid fermented (6), a molecular weight of 100 for an average amino acid, a fluid dilution rate of 0.07 h^{-1} (35), and a ruminal volume of 70,000 ml (35) are used, the additional loss of amino acids due to *C. aminophilum* is: 0.014 (1.1 mg of bacterial protein per ml)/(3.3 mg of protein per mmol of amino acid) $\times$ (100 mg/mmol of amino acid) $\times$ 70,000 ml $\times$ 0.07 h^{-1} $\times$ 24 h = 54,879 mg or 55 g of amino acids per day. Since the total protein intake of the cows was 630 g per day, it appeared that *C. aminophilum* might be wasting approximately 9% of the feed protein.

For more than 25 years, ruminant nutritionists have tried to "manipulate" ruminal fermentation with chemical additives (3), but the successes have often been "accidental" rather than "fortuitous." For example, monensin, the most reliable tool for manipulating ruminal fermentation, was originally developed as a coccidiostat for chickens (27). The studies on ruminal fermentation were preceded by animal trials that had already shown a positive effect, and the impact of monensin on individual ruminal bacteria was initially ignored (27).

Truly mechanistic studies of ruminal ecology are confounded by the complexity of ruminal bacteria, the labor-intensive nature of traditional enumeration techniques, and the inability of rumen microbiologists to cultivate all of the ruminal bacteria. Many of these problems can be circumvented by 16S rRNA analysis techniques, but even these techniques do not negate the high cost of animal experimentation, animal variation, and the virtually infinite combinations of dietary ingredients. Simple in vitro systems (like the one described in this paper) do not mimic all aspects of ruminal fermentation but, if properly designed, can offer a mechanistic foundation for subsequent animal trials.

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