A Ribosomal RNA Approach for Assessing the Role of Obligate Amino Acid-Fermenting Bacteria in Ruminal Amino Acid Deamination

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Introduction

Amino acid deamination in the rumen is a nutritionally wasteful process that often produces more ammonia than the bacteria can utilize. 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. Because ammonia can be oxidized by nitrifying bacteria, nitrite and nitrate accumulate in ground water.

Most strains of ruminal bacteria produce little ammonia. Based on the activities and numbers in the rumen, *Prevotella* (*Bacteroides*) *ruminicola* seems to be the most important ammonia-producing bacterium. Later work, however, indicates that this species cannot account for all the ammonia accumulation in vivo.

Ruminal bacteria have been traditionally isolated in media containing carbohydrates and either ammonia, or low concentrations of protein hydrolyzate, as a nitrogen source. In the late 1980's, enrichments on a high concentration of protein hydrolyzate yielded three ruminal bacteria with very high specific activities on ammonia production, and these bacteria could not utilize carbohydrates as an energy source. 16S rRNA sequence analyses indicated that these obligate amino acid-fermenting, monensin-sensitive bacteria were *Peptostreptococcus anaerobius, Clostridium sticklandii* and a new species designated as *Clostridium aminophilum*.

Most probable number estimates indicated that these monensin-sensitive, obligate amino acid-fermenting bacteria were less than 10% of the total ruminal count. 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. Because 16S rRNA can differentiate bacteria without in vitro culturing, we used 16S rRNA probes to assess the contribution of obligate amino acid-fermenting bacteria in vitro and in vivo.

Materials and Methods

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 two hours. Monensin was added to the diet to achieve a daily dose of 350 mg/day. Ruminal bacteria were grown anaerobically at 39°C in a basal medium containing salts, cysteine, sulfide, vitamins and minerals, 2-mercaptoethanesulfonic acid, volatile fatty acids, Trypticase and carbohydrates.

Aligned 16S rRNA nucleic acid sequences were obtained from the Ribosomal Data Base and probe sequences that had at least 3 to 4 mismatches in comparison to their closest phylogenetic relatives were chosen. rRNA was extracted by bead-beating and denatured by glutaraldehyde. The rRNA sample was diluted with polyadenylic acid and bromophenol blue, and applied to nylon membranes in a slot-blot apparatus. Membranes were air dried and then baked at 120°C for 30 min. Samples were prehybridized at room temperature for 2 h. Digoxygenen labeled probes were added to the hybridization solution and allowed to incubate at 22°C for 16 h. The relative abundance of 16S rRNA was determined with a universal probe.

Ammonia was analyzed by the indophenol method. Cell protein was measured by the folin method. Fermentation acids and carbohydrates were determined by high-performance liquid chromatography.

Results and Discussion

The 16S rRNA probes indicated that monensin inhibited the growth of *P. anaerobius* and *C. sticklandii* in the rumen. *C. 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 2-fold 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 the 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. Based on these results, the feed additive, monensin, cannot entirely counteract the wasteful amino acid deamination of obligate amino acid-fermenting ruminal bacteria.

The monensin-resistance of *C. aminophilum* reduces the amino acid sparing effect of monensin. Based on a bacterial percentage of 1.4, a bacterial protein concentration of 1.1 mg/ml, a yield for *C. aminophilum* of 3.3 mg protein/mmol amino acid fermented, a molecular weight of 100 for an average amino acid, a fluid dilution rate of 0.07 h^{-1}, a ruminal volume of 70,000 ml, the additional loss of amino acids due to *C. aminophilum* would be 55 g of amino acids/day. Since the total protein intakes of the cows were 630 g/day, it appeared that *C. aminophilum* might be wasting approximately 9% of the feed protein.

For more than 25 years, ruminant nutritionists tried to "manipulate" ruminal fermentation with chemical additives, but the successes have often been accidental. Truly mechanistic studies of ruminal ecology are confounded by the complexity of ruminal bacteria, the labor intensity 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 analyses, but even these techniques do not negate the high cost of animal experimentation, animal variation and the virtually infinite combination of dietary ingredients.

Conclusions

In vitro systems do not mimic all aspects of ruminal fermentation but if properly designed can offer a mechanistic foundation for subsequent animal trials.