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Ability of *Acidaminococcus fermentans* To Oxidize *trans*-Aconitate and Decrease the Accumulation of Tricarballylate, a Toxic End Product of Ruminal Fermentation

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Mixed ruminal bacteria convert *trans*-aconitate to tricarballylate, a tricarboxylic acid which chelates blood divalent cations and decreases their availability (J. B. Russell and P. J. Van Soest, Appl. Environ. Microbiol. 47:155–159, 1984). Decreases in blood magnesium in turn cause a potentially fatal disease known as grass tetany. *trans*-Aconitate was stoichiometrically reduced to tricarballylate by *Selenomonas ruminantium*, a common ruminal bacterium in grass-fed ruminants (J. B. Russell, Appl. Environ. Microbiol. 49:120–126, 1985). When mixed ruminal bacteria were enriched with *trans*-aconitate, a *trans*-aconitate-oxidizing bacterium was also isolated (G. M. Cook, F. A. Rainey, G. Chen, E. Stackebrandt, and J. B. Russell, Int. J. Syst. Bacteriol. 44:576–578, 1994). The *trans*-aconitate-oxidizing bacterium was identified as *Acidaminococcus fermentans*, and it converted *trans*-aconitate to acetate, a nontoxic end product of ruminal fermentation. When *S. ruminantium* and *A. fermentans* were cocultured with *trans*-aconitate and glucose, tricarballylate never accumulated and all the *trans*-aconitate was converted to acetate. Continuous-culture studies (dilution rate, 0.1 h^{-1}) likewise indicated that *A. fermentans* could outcompete *S. ruminantium* for *trans*-aconitate. When mixed ruminal bacteria were incubated in vitro with 10 mM *trans*-aconitate for 24 h, 45% of the *trans*-aconitate was converted to tricarballylate. Tricarballylate production decreased 50% if even small amounts of *A. fermentans* were added to the incubation mixes (0.01 mg of protein per mg of mixed bacterial protein). When *A. fermentans* (2 g of bacterial protein) was added directly to the rumen, the subsequent conversion of *trans*-aconitate to tricarballylate decreased 50%, but this effect did not persist for more than 18 h. *A. fermentans* eventually washed out of the rumen because the grass hay diet fed to the cow did not contain energy sources which could be utilized by *A. fermentans*. Because of its rapid rate of *trans*-aconitate oxidation and ability to compete with other ruminal bacteria for *trans*-aconitate, *A. fermentans* may have potential for preventing the sudden increases in tricarballylate levels which are associated with grass tetany.

Grass tetany, a potentially fatal disease of mature ruminants, has been recognized since the 1930s, but the etiology of the hypomagnesemia was perplexing. In the 1960s, Burau and Stout (3) found a strong correlation between the *trans*-aconitic acid content of grasses and the incidence of this disease. From the capacity of *trans*-aconitate to chelate magnesium, it appeared that *trans*-aconitate was decreasing magnesium absorption (9). This hypothesis was supported by the demonstration that oral doses of *trans*-aconitate could decrease blood magnesium levels (2).

The direct involvement of *trans*-aconitic acid in grass tetany was, however, contradicted by the observation that *trans*-aconitate had a short half-life in ruminal fluid (11). Subsequent work showed that ruminal bacteria rapidly converted *trans*-aconitate to tricarballylate, another tricarboxylic acid (19). Both sheep given *trans*-aconitate (17) and cattle fed grasses containing *trans*-aconitic acid (18) absorbed tricarballylate but not *trans*-aconitate. Rat studies showed that tricarballylic acid could increase the excretion of magnesium and other divalent cations by chelating blood magnesium (21).

trans-Aconitate was stoichiometrically converted to tricarballylate by *Selenomonas ruminantium*, a common bacterium in grass-fed ruminants (15). Mixed ruminal bacteria also converted some of the *trans*-aconitate to acetate, a normal and nontoxic end product of ruminal fermentation, but the organism(s) responsible for this conversion was not initially isolated (15). The conversion of *trans*-aconitic acid to acetic acid was decreased by methane inhibitors, and this result indicated that hydrogen was a likely end product of *trans*-aconitic acid oxidation (17).

Ruminal enrichment cultures with *trans*-aconitate yielded a bacterium that was subsequently identified as *Acidaminococcus fermentans* (7). *A. fermentans* converted *trans*-aconitate to acetate, carbon dioxide, and hydrogen (7), but its ability to compete with *S. ruminantium* for *trans*-aconitate was not known. The following experiments were designed to assess the ability of *A. fermentans* to compete with *S. ruminantium* in vitro, persist in the rumen, and decrease the conversion of *trans*-aconitate to tricarballylate.

MATERIALS AND METHODS

Cell growth in vitro. *A. fermentans* AO was grown anaerobically at 39°C in basal medium containing (per liter) 292 mg of K_2HPO_4, 292 mg of KH_2PO_4, 480 mg of Na_2SO_4, 480 mg of NaCl, 100 mg of $MgSO_4 \cdot 7H_2O$, 64 mg of $CaCl_2 \cdot 2H_2O$, 600 mg of cysteine hydrochloride, vitamins, macrominerals (8), 1 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), and 20 mmol of *trans*-aconitate. When *S. ruminantium* HD4 was grown, the basal medium was supplemented with 0.5 g of yeast extract, 50 μg of biotin, and 1 mmol of valerate per liter, and glucose (5.5 mM) was the energy source. The medium was adjusted to pH 6.7, and the final pH was never less than 6.5 at

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the end of growth. For all batch growth experiments, cells were grown anaerobically in 80-ml serum bottles which were capped with butyl rubber stoppers and aluminum seals, and the optical density at 600 nm was monitored. Coculture batch experiments were performed with supplemented medium containing 10 mM *trans*-aconitate and 1 g of glucose per liter. Experiments were initiated by the addition of *A. fermentans* and *S. ruminantium* HD4 cultures (approximately 0.2 mg of protein of each) that had been grown on 10 mM *trans*-aconitate and *trans*-aconitate plus glucose, respectively.

S. ruminantium was also grown in a glucose-limited (5.5 mM) continuous culture that contained 10 mM *trans*-aconitate (360-ml culture vessel; dilution rate, 0.1 h^{-1}; pH 6.7). The continuous culture reached a steady state in 48 h. An inoculum of *A. fermentans* (2 μg of protein per ml) was then added to the culture vessel, and the competition between *S. ruminantium* and *A. fermentans* was monitored visually (phase contrast microscope at ×1,250 magnification) and by the relative conversion of *trans*-aconitate to either tricarballylate or acetate. Cells were separated from culture fluid by centrifugation (10,000 × *g*, 5°C, 10 min), and cells were washed twice with 0.9% NaCl. Cells and cell-free culture fluid were stored at −15°C.

Ruminal studies. A 600-kg nonlactating Holstein dairy cow was fed 8 kg of grass hay per day with a rotary carousel feeder (one meal every 2 h). The grass hay contained 11.1% crude protein, 69.1% neutral detergent fiber, 47.1% acid detergent fiber, 0.24% magnesium, and 0.78% calcium (dry matter basis). The cow had an 80-liter rumen, and the fluid dilution rate was 0.06 h^{-1}.

Ruminal fluid samples were obtained from five locations in the rumen with a suction tube. The ruminal fluid was incubated anaerobically for 30 min at 39°C. Once the large feed particles were buoyed to the top of the flask by gas accumulation, a particle-free sample was obtained from the center of the flask. The particle-free sample containing mixed ruminal microorganisms was then transferred in triplicate (33%, vol/vol) to basal medium containing 10 mM *trans*-aconitate. After 24 h of incubation at 39°C, the cells were separated from the culture fluid and stored (see above).

A. fermentans was cultured in basal medium containing 10 mM *trans*-aconitate (10 liters, 39°C), and the cells were harvested anaerobically by centrifugation (10,000 × *g*, 30 min, 15°C). The cells (approximately 2 g of cell protein) were transported to the cow in an anaerobic flask, and the cells were placed at five sites in the rumen. The ruminal fluid was sampled, and triplicate cultures were monitored for *trans*-aconitate fermentation as described above. The ruminal inoculation with *A. fermentans* was performed twice.

Other analyses. Optical density was monitored in 1-cm cuvettes at 600 nm. Protein from NaOH-hydrolyzed cells (0.2 M NaOH, 100°C, 15 min) was assayed by the method of Lowry et al. (12). Hydrogen gas was measured with a Gow-Mac model 550 thermal conductivity gas chromatograph with a Supelco S-8100 column. Fermentation acids in the cell-free supernatant samples were analyzed by high-pressure liquid chromatography with a Beckman 334 liquid chromatograph which was equipped with a model 156 refractive index detector and a Bio-Rad HPX-87H organic acid column. The sample size was 20 μl, the elutant was 0.0065 M H_2SO_4, the flow rate was 0.5 ml/min, and the column temperature was 50°C.

Statistics. All experiments were performed two or more times. The coefficient of variation for the in vitro growth and competition experiments was less than 10%. The ruminal inoculation experiments were performed twice, and the mean and standard error are given.

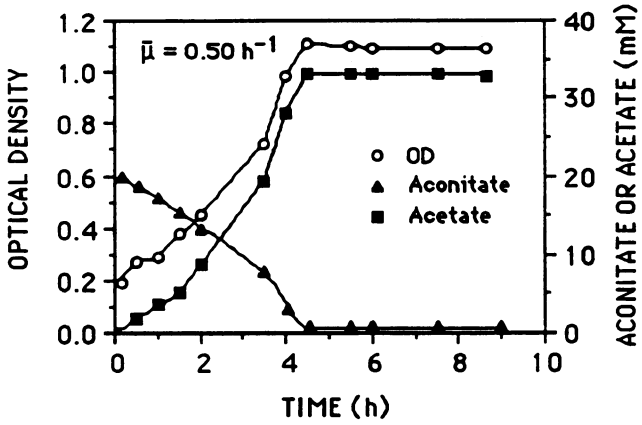

FIG. 1. Growth of and acetate production by *A. fermentans* on *trans*-aconitate. OD, optical density.

RESULTS

Growth of *A. fermentans*. *A. fermentans* converted each mole of *trans*-aconitate to 2 mol of acetate, and its growth rate on *trans*-aconitate was 0.5 h^{-1} (Fig. 1). Hydrogen was also detected. The growth yield was 12.1 mg of protein per mmol of *trans*-aconitate. No growth was detected with tricarballylate as the sole carbon and energy source.

Growth of *S. ruminantium*. *S. ruminantium* HD4 could not utilize *trans*-aconitate as a sole energy source for growth but converted *trans*-aconitate to tricarballylic acid when glucose was provided as an energy source (Fig. 2a and b). Lactate was the initial end product of glucose fermentation, but the lactate was subsequently converted to acetate and propionate when the glucose was depleted (Fig. 2b). Tricarballylate accumulation occurred before and after glucose depletion (Fig. 2c). The stoichiometry of *trans*-aconitate utilization and tricarballylic acid accumulation was 1:1. Tricarballylic acid was never detected unless *trans*-aconitate was provided.

Cocultures of *A. fermentans* and *S. ruminantium*. When a batch culture medium containing glucose and *trans*-aconitate was inoculated with *A. fermentans* and *S. ruminantium* (approximately 0.2 mg of protein of each), the coculture grew at a specific rate of 0.73 h^{-1}, and both cell types (diplococci and crescent shaped) were observed (Fig. 3a). When growth ceased at 6 h, lactate and acetate were the only fermentation end products, and tricarballylate could not be detected (Fig. 3b and c). Even after 24 h, there was no decrease in the lactate level or accumulation of acetate, propionate, or tricarballylic acid. The final pH was 6.4.

When *S. ruminantium* was grown anaerobically in a glucose-limited chemostat for 2 days with 9 mM *trans*-aconitate, propionate, acetate, and tricarballylate were the fermentation end products, and only one-third of the *trans*-aconitate was utilized (Fig. 4). When *A. fermentans* was added to the chemostat at time zero, the remaining *trans*-aconitate was utilized and acetate production increased. The addition of *A. fermentans* eventually caused a decrease in the tricarballylate level, and this decrease was associated with an increase in the propionate level. By 6 days, little if any tricarballylate could be detected. From the optical density and fermentation end products, it appeared that the relative numbers of *A. fermentans* and *S. ruminantium* remained constant between 2 and 6 days.

Competition of *A. fermentans* with mixed ruminal bacteria in vitro. When mixed ruminal bacteria were incubated with 10

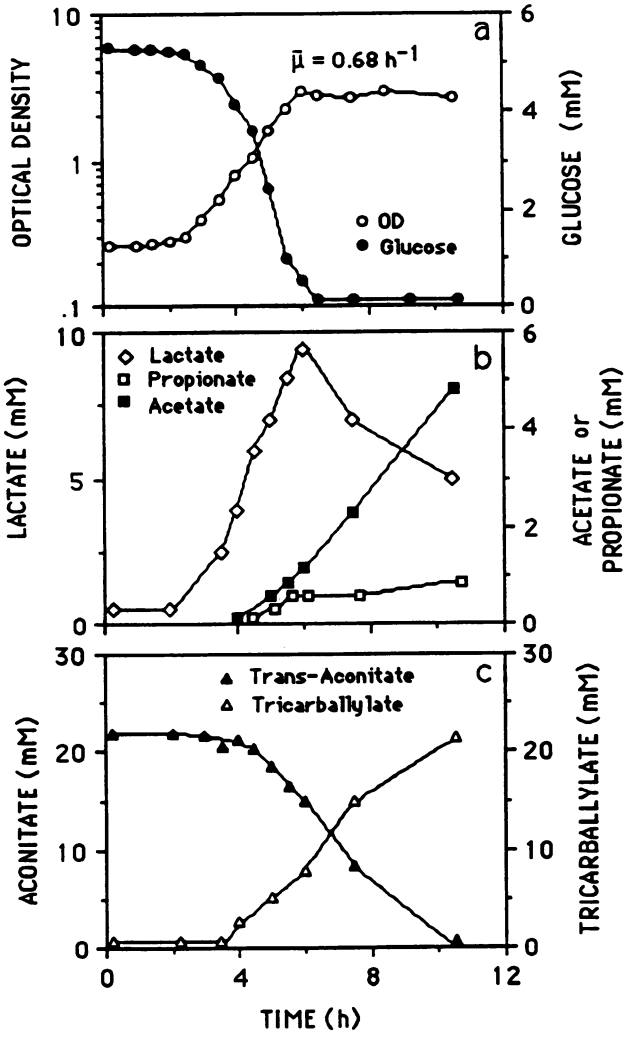

FIG. 2. *S. ruminantium* grown on glucose. (a) Growth; (b) lactate, acetate, and propionate production; (c) conversion of *trans*-aconitate to tricarballylate.

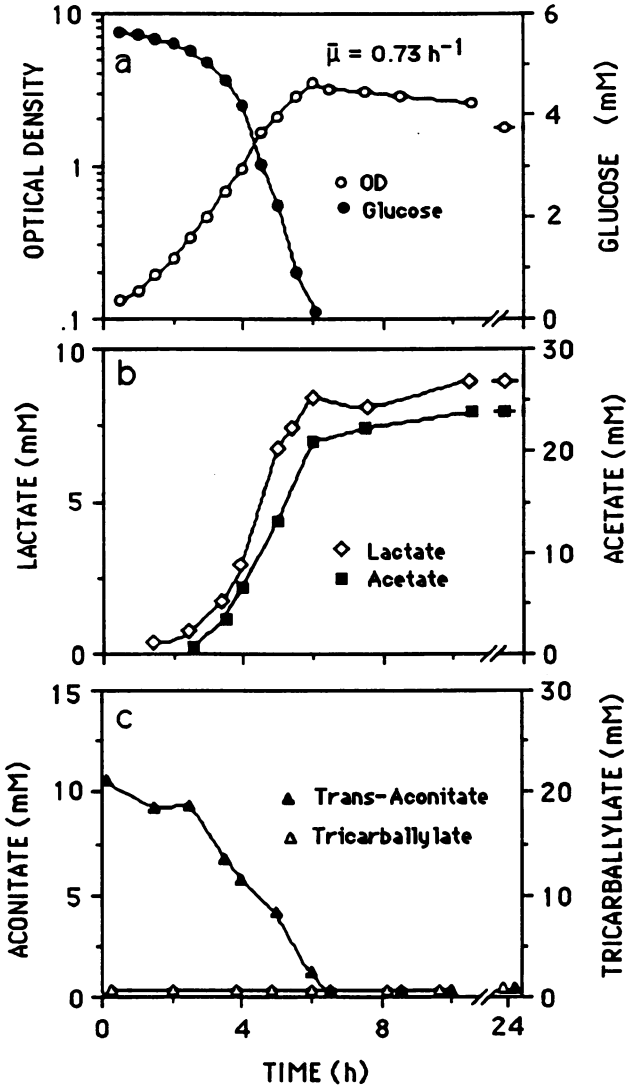

FIG. 3. Coculture of *A. fermentans* and *S. ruminantium* grown on glucose and *trans*-aconitate. (a) Growth; (b) lactate and acetate production; (c) conversion of *trans*-aconitate to tricarballylate.

mM *trans*-aconitate for 24 h in vitro, all of the *trans*-aconitate was utilized and approximately 45% of the *trans*-aconitate was converted to tricarballylate (Fig. 5). The addition of *A. fermentans* to the mixed ruminal bacteria decreased tricarballylate accumulation even when the ratio of *A. fermentans* protein to mixed ruminal bacteria protein was as low as 1 to 100. Higher ratios of *A. fermentans* to the mixed ruminal bacteria were, however, not able to completely eliminate tricarballylate accumulation.

Tricarballylate production by ruminal fluid from a cow inoculated with *A. fermentans*. When *A. fermentans* (2 g of bacterial protein) was added to the rumen of a fistulated cow, there was an immediate decrease in the capacity of the mixed ruminal bacteria to convert *trans*-aconitate to tricarballylate (Fig. 6). Prior to inoculation, approximately 45% of the *trans*-aconitate was converted to tricarballylate, and this conversion rate was decreased to 20% after the addition of *A. fermentans*. However, the decrease in tricarballylate accumulation did not persist for more than 18 h.

DISCUSSION

Acute cases of grass tetany in cattle are usually observed in the spring when there is rainfall, an increase in temperature, rapid plant growth, an increase in plant potassium, and an accumulation of *trans*-aconitate, which serves as an anion for potassium (1). Grasses belonging to the wheat family can accumulate as much as 6% *trans*-aconitate (3, 13, 14, 22), and *trans*-aconitate concentrations of greater than 1% are considered toxic (9). Animal losses on wheat pasture were commonly 2 to 3%, but losses as great as 20% have been reported (1).

Because ruminal bacteria rapidly reduce *trans*-aconitate to tricarballylate (17, 19), it appeared that the ruminal accumulation of tricarballylate rather than *trans*-aconitate per se was causing the problem. This hypothesis is supported by the observations that animals absorb tricarballylate but not *trans*-aconitate (18) and that tricarballylate causes increased urinary loss of divalent cations (21).

trans-Aconitate is converted to tricarballylate by *Clostridium*

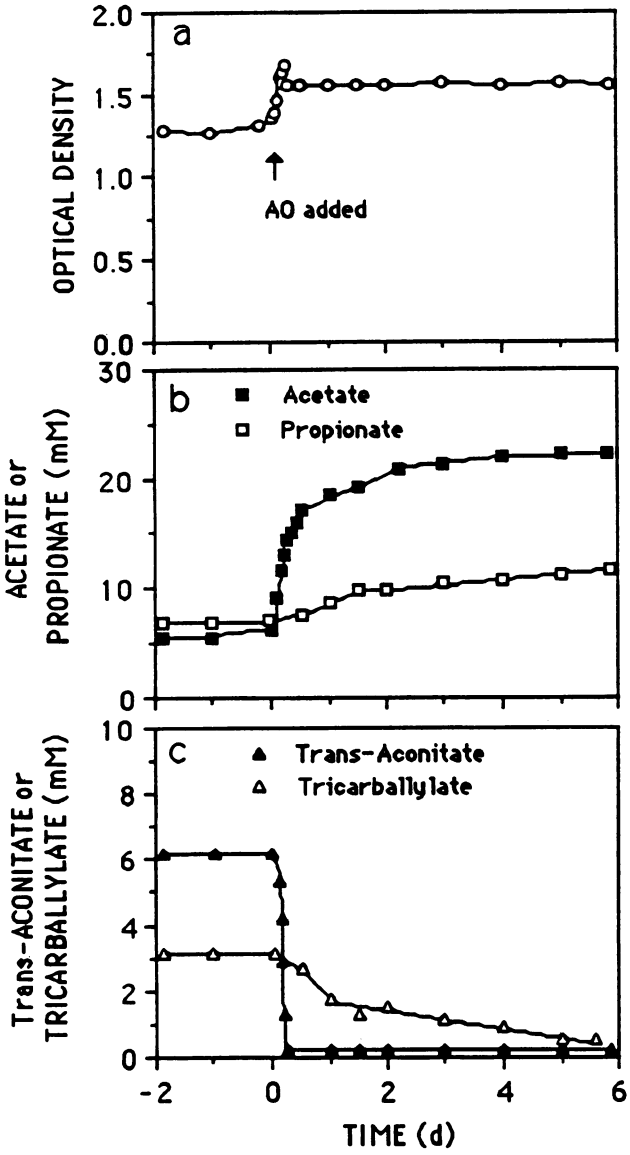

FIG. 4. Growth of *S. ruminantium* on glucose and *trans*-aconitate in continuous culture (dilution rate, 0.1 h^{-1}; pH 6.7) and the effect of *A. fermentans* AO inoculation (at time zero) on growth (a), acetate and propionate production (b), and conversion of *trans*-aconitate to tricarballylate (c). The reservoir concentrations of *trans*-aconitate and glucose were 9 and 5.5 mM, respectively.

clostridiiforme, a spiral bacterium, *Wolinella succinogenes*, and *S. ruminantium* (15), but only *S. ruminantium* is a common ruminal bacterium (10). *S. ruminantium* has a high affinity for sucrose (16), and grasses which accumulate *trans*-aconitate often have high concentrations of sucrose (18). *S. ruminantium* does not utilize *trans*-aconitate as an energy source, but it converts *trans*-aconitate stoichiometrically to tricarballylate when sugar is provided as the energy source for growth (Fig. 2).

S. ruminantium's fermentation is homolactic in batch culture, but lactate is subsequently converted to propionate and acetate when the sugar is depleted (20). In low-dilution-rate continuous cultures, propionate and acetate are the primary

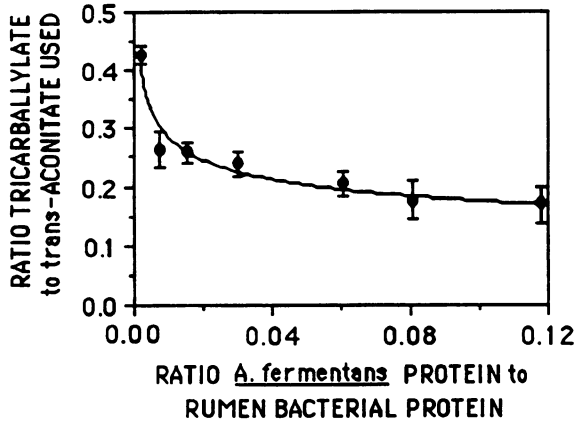

FIG. 5. Effect of *A. fermentans* on the conversion of *trans*-aconitate (10 mM) to tricarballylate by mixed ruminal bacteria incubated for 24 h in vitro.

end products of glucose fermentation, and the ratio of these acids is typically 2 to 1, respectively (20). Because *trans*-aconitate utilization by *S. ruminantium* is a reducing equivalent disposal mechanism, it should cause a decrease in the ratio of propionate to acetate. When *trans*-aconitate was added to batch cultures of *S. ruminantium*, little *trans*-aconitate was used during the homolactic phase of growth, but *trans*-aconitate utilization subsequently caused a decrease in the propionate and an increase in the acetate level (Fig. 2). A decrease in propionate production was also observed in continuous culture (Fig. 4).

A. fermentans grew very rapidly with *trans*-aconitate as an energy source, and coculture experiments indicated that it could compete with *S. ruminantium* for *trans*-aconitate. When *A. fermentans* and *S. ruminantium* were incubated in batch culture, tricarballylate never accumulated and all of the *trans*-aconitate was converted to acetate. Previous work by Chen and Wolin (6) indicated that conversion of lactate to acetate and propionate by *S. ruminantium* is dependent on the removal of hydrogen. Because *A. fermentans* produces a considerable

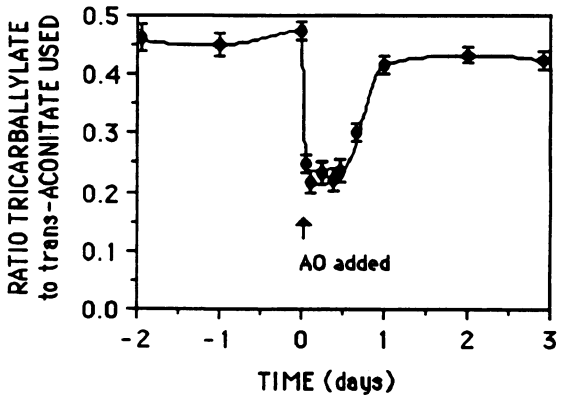

FIG. 6. Effect of *A. fermentans* AO addition (2 g of bacterial protein) to the rumen on subsequent conversion of *trans*-aconitate (10 mM) to tricarballylate by ruminal bacteria. Mixed ruminal bacteria were obtained from the rumen and incubated for 24 h in vitro with 10 mM *trans*-aconitate. The ruminal inoculation was performed twice, and the mean results (± the standard error) are shown.

amount of hydrogen gas (7), it is not surprising that the *A. fermentans-S. ruminantium* coculture left more lactate in the medium than the *S. ruminantium* monoculture (compare Fig. 3 with Fig. 2).

A continuous culture of *A. fermentans* and *S. ruminantium* initially produced some tricarballylate, but tricarballylate production was eventually eliminated. Because the addition of even a small inoculum of *A. fermentans* to mixed ruminal bacteria in vitro decreased tricarballylate accumulation by 50%, it appeared that *A. fermentans* was able to survive in a mixed ruminal environment and compete with other ruminal bacteria for *trans*-aconitate. This assumption was supported by the observation that the addition of only 2 g of *A. fermentans* protein to the rumen also caused an immediate decrease in the capacity of the ruminal fluid to convert *trans*-aconitate to tricarballylate.

The ecological niche of *A. fermentans* in the rumen is not entirely clear. When mixed ruminal bacteria from cows not consuming *trans*-aconitate are incubated with *trans*-aconitate in vitro, more than 50% of the *trans*-aconitate is converted to products other than tricarballylate (17, 19) (Fig. 5). Because the concentration of *A. fermentans* in the rumen was only 10^2/ml (7) and that of mixed ruminal bacteria is greater than 10^{10}/ml (4), it appears that other bacteria are also able to oxidize *trans*-aconitate. The success of *A. fermentans* in sequential *trans*-aconitate enrichments and batch culture incubations is most easily explained by its rapid rate of *trans*-aconitate catabolism and ability to utilize *trans*-aconitate as a sole energy source for growth.

Tricarballylate production was only transiently decreased by ruminal inoculation of *A. fermentans*. The dilution of *A. fermentans* from the rumen is probably related to its relatively narrow range of potential substrates (7). The grass hay which was fed to the cow did not contain significant amounts of *trans*-aconitate, citrate, or pyruvate. The hay did contain the amino acid glutamate, another potential energy source for *A. fermentans*, but this amino acid is rapidly deaminated by mixed ruminal bacteria (5). Since *trans*-aconitate accumulation in plants is a transient and somewhat unpredictable event that is observed only in grazing situations (9, 13, 14) and commercial *trans*-aconitate is expensive, it was not possible for us to feed the cow a diet which contained *trans*-aconitate.

The inoculation of natural habitats with bacteria has usually been confounded by the ubiquitous nature of bacteria and the dependence of all bacteria on a suitable niche. These general principles of microbial ecology, however, do not account for sudden and transient changes in substrate availability. Grasses accumulate *trans*-aconitate very rapidly, and there is often little *trans*-aconitate prior to the accumulation (14). By the time that *A. fermentans* would be able to grow to a significant density, the cow has already been exposed to a lethal accumulation of tricarballylate. Further work is needed to see whether ruminal inoculation of *A. fermentans* can provide a means of decreasing tricarballylate accumulation and mortality.

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