Enrichment and Isolation of Rumen Bacteria That Reduce \textit{trans}-Aconitic Acid to Tricarballylic Acid

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Bacteria from the bovine rumen capable of reducing \textit{trans}-aconitie acid to tricarballylate were enriched in an anaerobic chemostat containing rumen fluid medium and aconitate. After 9 days at a dilution rate of 0.07 h\(^{-1}\), the medium was diluted and plated in an anaerobic glove box. Three types of isolates were obtained from the plates (a crescent-shaped organism, a pleomorphic rod, and a spiral-shaped organism), and all three produced tricarballylate in batch cultures that contained glucose and \textit{trans}-aconitie. In glucose-limited chemostats (0.10 h\(^{-1}\)), \textit{trans}-aconitie reduction was associated with a decrease in the amount of reduced products formed from glucose. The crescent-shaped organism produced less propionate, the pleomorphic rod produced less ethanol, and the spiral made less succinate and possibly H\(_2\). Aconitie reduction by the pleomorphic rod and the spiral organism was associated with a significant increase in cellular dry matter. Experiments with stock cultures of predominant rumen bacteria indicated that \textit{Selenomonas ruminantium}, a species taxonomically similar to the crescent-shaped isolate, was an active reducer of \textit{trans}-aconitie. Strains of \textit{Bacteroides ruminicola}, \textit{Butyrivibrio fibrisolvens}, and \textit{Megasphaera elsdonii} produced little if any tricarballylate. \textit{Wolinella succinogenes} produced some tricarballylate. Based on its stability constant for magnesium (\(K_{eq} = 115\)), tricarballylate could be a factor in the hypomagnesemia that leads to grass tetany.

When plants are grown under cloudy conditions, with high levels of nitrogen fertilization, organic acids and, in particular, \textit{trans}-aconitic acid can accumulate (9, 12, 19). \textit{Trans}-aconitic acid, in severe conditions, can account for more than 4\% of the total dry matter (23), and high levels of this acid were associated with toxic responses in ruminant animals (2, 3, 6, 11, 12). Stout et al. suggested that \textit{trans}-aconitie acid could form chelates with magnesium and decrease the availability of dietary magnesium (23). Bohman et al. subsequently showed that oral administration of \textit{trans}-aconitic acid could induce symptoms of hypomagnesemia, commonly termed grass tetany (3).

Recent experiments indicated that \textit{trans}-aconitic acid was rapidly fermented by mixed rumen bacteria in vitro, and it seemed unlikely that \textit{trans}-aconitic acid would be present in rumen fluid for a long enough time to decrease the availability of dietary magnesium (22). \textit{Trans}-aconitic acid fermentation, however, led to an accumulation of acetic acid and an unknown compound that was subsequently identified as tricarballylic acid (Fig. 1 [22]). Tricarballylic acid was fermented very slowly by mixed rumen bacteria. Based on its three exposed carboxyl groups, it appeared that tricarballylic acid might be an important factor in the hypomagnesemia that leads to grass tetany.

The following experiments describe the enrichment, isolation, and characteristics of \textit{trans}-aconitic acid-reducing rumen bacteria. Several common strains of rumen bacteria were also tested for the capacity to reduce \textit{trans}-aconitic acid to tricarballylic acid.

\section*{Materials and Methods}

\subsection*{Enrichment and isolation.} An anaerobic chemostat (360-ml culture vessel, O\(_2\)-free CO\(_2\) gas phase, 0.07 h\(^{-1}\) dilution rate) was inoculated with mixed rumen bacteria from a cow fed timothy hay. The medium reservoir contained (in milligrams per liter): K\(_2\)HPO\(_4\), 292; KH\(_2\)PO\(_4\), 292; (NH\(_4\))\(_2\)SO\(_4\), 480; NaCl, 480; MgSO\(_4\) \cdot 7H\(_2\)O, 100; CaCl\(_2\) \cdot 2H\(_2\)O, 64; NaCO\(_3\), 1,000; cysteine hydrochloride, 600; hemin, 1; pyridoridinamine dihydrochloride, 2; riboflavin, 2; thiamine hydrochloride, 2; nicotinamide, 2; calcium pantothenate, 2; lipoic acid, 1; \textit{para}-aminobenzoic acid, 0.1; folic acid, 0.05; biotin, 0.05; coenzyme B\(_12\), 0.05; valeric acid, 100; isovaleric acid, 100; isobutyric acid, 100; 2-methylbutyric acid, 100; \textit{trans}-aconitate, 13.5 mmol; and clarified rumen fluid, 10\% (vol/vol) (see references 20 and 21 for method of preparation). The \textit{trans} isomer of aconitic acid was obtained from Sigma Chemical Co., St. Louis, Mo., and hereafter is referred to simply as aconitic acid.

Each day samples were taken from the culture vessel, and optical density, pH, and organic acids were analyzed (methods described below). On day 9, the culture was serially diluted (10-fold increments) with sterile medium (same as chemostat medium) that contained 2\% molten agar (47\(^\circ\)C) and 0.2\% carbohydrates (equal parts glucose, maltose, and cellobiose) or no added carbohydrates. After 48 h of incubation (39\(^\circ\)C) in an anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.), the plates were examined for growth. When carbohydrates were provided, colonies were present up to the 10\(^{th}\) dilution. Plates not containing carbohydrates also had colonies, but these colonies were much smaller. Fifteen isolated colonies from each set of plates were picked and inoculated into medium plus 0.2\% glucose. All 30 isolates grew on glucose. Broth cultures were replated, picked, and examined microscopically (\(\times1,250\)) for purity. Three cell types were observed (pleomorphic rods, crescent-shaped cells, and spiral organisms). Each of the strains was then inoculated in medium with different carbohydrates, lactate, and mannitol (Table 1). All of the strains within a cell type exhibited the same pattern of growth, and one strain from each cell type was retained.

\subsection*{Fermentation.} The pleomorphic rod (strain D), the crescent-shaped cells (strain A), and the spiral organism (strain M) were then grown in rumen fluid medium (see above) which was supplemented with glucose (2 g/liter). Strains D
and M grew slowly in the medium and were provided with an additional 4.0 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.) and 4.0 g of yeast extract per liter. After these additions, all three strains grew well in the medium. The strains were also grown in continuous culture (see above). In this case, the medium reservoir contained rumen fluid medium (see above), 4.0 g of Trypticase per liter, and 4.0 g of yeast extract per liter. Each strain was grown with and without aconitic acid (approximately 7 mM) to ascertain the effect of this acid on fermentation products.

Other rumen bacteria. A variety of stock cultures were likewise tested for the ability to reduce aconitate to tricarballylate. Bacteroides ruminicola B4, Butyribrio fibrisolvens A38 and 49, Megasphaera elsdenii T81 and B159, and Selenomonas ruminantium HD4 were obtained from M. P. Bryant, University of Illinois, Urbana. B. ruminicola 23 and S. ruminantium D were provided by K. A. Dawson, University of Kentucky, Lexington. Streptococcus bovis 45S1 was obtained from C. S. Stewart, Rowett Research Institute, Scotland, and T. L. Miller, New York State Department of Health, Albany, provided Wolinella succinogenes. All strains were grown in basal medium that contained aconitate (10 mM), Trypticase (4.0 g/liter), and yeast extract (4.0 g/liter).

Analyses. Optical density was measured with a Gilford model 260 spectrophotometer (600 nm and cuvets of 1-cm light path). Volatile fatty acids, glucose, lactate, succinate, aconitate, and tricarballylate were analyzed by high-pressure liquid chromatography (Beckman model 334 liquid chromatograph, model 156 refractive index detector, model 421 CRT data controller, CRI A integrator, 50-μ loop, 50°C), using a Bio-Rad HPX-87H organic acid column (see references 10 and 22). Ethanol was determined by an enzymatic procedure (4), and ammonia was assayed by the method of Chaney and Marbach (8). Cysteine interference with ammonia color formation was minimized by using six times as much reagent. All analyses were performed in duplicate, and the variation between replicates was always <7%.

**RESULTS**

When mixed rumen bacteria were inoculated into a chemostat that contained rumen fluid medium and aconitate, there was a marked decrease in optical density between days 0 and 4 (Fig. 2). Soon after inoculation virtually all of the available aconitate was degraded and acetate and propionate were the primary fermentation products. Between days 2 and 5, aconitate utilization declined and there was an increase in tricarballylate. From day 5 to 9, optical density and the rate of aconitate utilization once again increased. Tricarballylate also increased during this time period and there was little increase in acetate and propionate until day 8. pH was nearly neutral throughout the incubation, and recovery of carbon from aconitic acid as acetic, propionic, or tricarballylic acids ranged from 53 to 86% on days 1 to 4 and from 87 to 102% on days 5 to 9.

On day 9 of continuous culture, samples were withdrawn from the culture vessel, diluted, and plated on rumen fluid media that contained either aconitate or aconitate and carbohydrates. After 48 h of incubation, both plate types had colonies at the 10⁶ dilution, but colonies from plates with only aconitate were much smaller. Isolated colonies were transferred to rumen fluid media that contained glucose, and growth was observed in all cases. Isolates were then examined microscopically and screened for energy source utilization and fermentation products (Table 1). Three types of

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* C. Crescent shaped; R. rods; S. spiral organisms.
+ High turbidity; –, no turbidity; ±, small amount of turbidity after 24 or 48 h of growth with 2.0 g of the energy source per liter.
Fermentation products formed in batch culture with 2.0 g of glucose per liter.
Tricarballylate was only a product when aconitic acid was added to the medium.
organisms were identified and duplicate strains were discarded.

The crescent-shaped organism (Fig. 3a) was able to ferment a variety of hexoses, pentoses, disaccharides, starch, and mannitol (Table 1). It was unable to grow on cellulose, lactate, or malate. When grown on glucose in batch culture, it was motile and produced lactate, acetate, and propionate. The pleomorphic rod (Fig. 3b) was also able to grow on glucose, fructose, arabinose, sucrose, and cellobiose (Table 1). When mannose, xylose, lactose, and starch were provided, final turbidity was low (<0.3 optical density unit) even after 48 h of incubation. This motile rod produced formate, acetate, and ethanol in batch culture. The spiral-shaped organism (Fig. 3c) was only able to grow on glucose, fructose, mannose, and mannitol and produced succinate and acetate. All three organisms produced tricarballylate when aconitate was added to glucose rumen fluid medium. Tricarballylate was not formed in the absence of aconitate.

When the crescent-shaped organism (strain A) was grown in batch culture with glucose and aconitate, glucose was rapidly fermented and lactate was the primary product (Fig. 4a). Aconitate was also depleted and this degradation was associated with a nearly equal molar increase in tricarballylate. After glucose depletion at 5 h, the rate of aconitate disappearance declined, and a significant amount of aconitate was left in the medium even after 50 h.

The pleomorphic rod (strain D) also grew rapidly on glucose, and aconitate was completely metabolized in less than 10 h (Fig. 4b). Once again, aconitate utilization was accompanied by an equal molar increase in tricarballylate. Acetate and formate were also formed during the period of glucose fermentation, and the molar ratio of these two acids was nearly 1:1.

Strain M (the spiral organism) grew at a slower rate, and glucose fermentation closely paralleled the degradation of aconitate (Fig. 4c). At approximately 12 h, both glucose and aconitate were depleted, and after this time there was little increase in acetate, succinate, or tricarballylate. Comparison of aconitate utilization and tricarballylate formation also indicated a one-to-one relationship.
To see what effect aconitate reduction to tricarballylate was having on fermentation patterns, each of the three isolates was grown in continuous culture, with and without aconitate (Table 2). When strain A was grown in continuous culture with glucose and no aconitate, propionate and acetate were the primary products, and less lactate was observed. Addition of aconitate caused a marked decline in propionate and an increase in lactate, acetate, and tricarballylate. In both cases, the oxidation-reduction state (O/R) of the substrates and that of the products were roughly equal. Carbon recovery ranged from 95 to 103%. Strain A was not greatly stimulated by the addition of yeast extract or Trypticase (see above), and it seems likely that glucose was the primary source of cell carbon.

When strain D was grown in continuous culture, formate, acetate, and ethanol were formed from glucose (Table 2). Aconitate addition caused an equal molar increase in tricarballylate and a reduction in ethanol formation. O/R balances were consistent but carbon recoveries were >100%.
Strain D was greatly stimulated by added yeast extract and Trypticase, and these values indicated that much of the cell carbon was derived from these sources and not from glucose.

Strain M fermented glucose to acetate and succinate (Table 2). Aconitate reduction to tricarballylate was associated with a small decline in succinate and an increase in acetate. O/R calculations indicated that the products were more oxidized than the substrates, and this would indicate that a highly reduced product (i.e., $H_2$) was not detected. To maintain a strictly anaerobic environment in the chemostat, the culture vessel was continuously purged with O$_2$-free CO$_2$. This continuous gas flow confounded gas measurements, and $H_2$ production was not determined. Aconitate reduction may have been associated with a decrease in $H_2$ production. Trace amounts of $H_2$ were detected in batch culture. The organism was greatly stimulated by yeast extract and Trypticase, and carbon recoveries indicated that much of the cell carbon could have been derived from these sources. Variations in $H_2$ production would not have an effect on carbon recovery.

None of the isolates was able to grow on aconitate alone, and it is probable that the presence of bacteria in the enrichment chemostat (Fig. 2) was at least partially dependent on trace amounts of energy source in sterile rumen fluid. When aconitate was added to glucose-limited chemostats, however, there was an increase in cell dry matter (Table 2). These increases were most significant with strains D and M and indicated that aconitate reduction was having a positive influence on the energetics of growth.

To ascertain whether other rumen bacteria were able to reduce aconitate to tricarballylate, various strains of rumen bacteria were grown in batch culture with glucose and aconitate (Table 3). $S$. ruminantium HD4 was able to degrade 9.5 mM aconitate, and 9.0 mM tricarballylate was formed. $S$. ruminantium D used less aconitate but the utilization was once again accompanied by an equal molar increase in tricarballylate. Strains of $B$. ruminicola, Butyrivibrio fibrisolvens, $M$. elsdenii, and Streptococcus bovis used little aconitate and produced trace or insignificant amounts of tricarballylate. $W$. succinogenes converted 2 mM aconitate to tricarballylate, which was not nearly as much as $S$. ruminantium.

**DISCUSSION**

Batch cultures of mixed rumen bacteria from cows fed either timothy hay or 60% concentrate were found to convert approximately 40% of the added aconitate to tricarballylate (22). When a chemostat containing rumen fluid medium and aconitate was inoculated with mixed rumen bacteria, all aconitate entering the culture vessel disappeared by day 1, but tricarballylate was not a major product (Fig. 2). As the medium entry continued, however, tricarballylate production increased, accounting for 45% of the aconitate utilization on day 8.

Isolates from day 9 of continuous culture all produced tricarballylate in batch culture when aconitate was added to the medium (Table 1), and the ratio of aconitate utilized/tricarballylate produced was approximately 1 (Fig. 4). Acetate was also a significant product in continuous culture, but none of the isolates produced significant amounts of acetate from aconitate (Table 2). The absence of organisms that fermented aconitate to acetate may have been related to the 5% $H_2$ content of the anaerobic glove box. A possible pathway of acetate conversion to acetate might proceed via citrate, oxaloacetate, pyruvate, and acetyl coenzyme A. In this case, $H_2$ could be a product, and the inhibition of $H_2$-producing species by high partial pressures of $H_2$ is well documented (25).
Based on motility, crescent-shaped morphology, Gram stain, energy source utilization, and fermentation products (Table 1), it appeared that strain A closely fits the taxonomy of the rumen bacterium S. ruminantium (13, 15). This pleomorphic rod (strain D) resembled a bacteroides but absence of either succinate or propionate indicated that it was not a normal rumen type (i.e., B. ruminicola, B. succinogenes, or B. amylophilus). Based on absence of either succinate or propionate, abundant gas formation, and motility, strain D resembled Clostridium clostridiiforme, a species previously designated as Bacteroides clostriformis subsp. girans (15). Taxonomic classification of the spiral organism, strain M, is not certain. The spiral-shaped morphology indicated that it is most likely a spirochete, but other tests are needed before a more precise identification can be made.

Many rumen bacteria produce lactate, ethanol, and H2 in pure culture, but these products are usually not detected in rumen fluid. An explanation of the apparent discrepancy between pure- and mixed-culture studies was first proposed by Hungate (15). He stated that the removal of hydrogen by methanogens would allow rumen bacteria to reoxidize reduced nucleotides and produce more acetate. Since this time, Wolin and his colleagues (25) have demonstrated that interspecies hydrogen transfer to methanogens can decrease the formation of reduced end products (i.e., lactate, propionate, succinate, ethanol, and H2).

Aconitate reduction to tricarboxylate in chemostat culture was associated with a change in fermentation products (Table 2). With strain A, there was a decrease in propionate and an increase in acetate. Acetate production was not affected in strain D, but there was a decline in ethanol. Only small changes in the fermentation pattern were noted with cultures of strain M in the presence of aconitate. Differences in O/R indicated that H2 may have been a product, and aconitate reduction could have been at the expense of H2 production. These changes in fermentation pathways increased the efficiency of glucose utilization and cell growth. Because tricarboxylate formation represents an alternative means of reducing-equivalent disposal, there could be an antagonism between tricarboxylate and methane production in vivo.

Formation of tricarboxylate from aconitate occurs by a simple reduction (Fig. 1), and similar reactions are known to occur in the rumen (1, 14, 16, 18, 24, 26). When rumen bacteria capable of reducing unsaturated bonds were tested for their ability to reduce aconitate, only S. ruminantium was highly active (Table 3). W. succinogenes was also active but low numbers in rumen contents (26) suggest that this organism would be less important.

The importance of tricarboxylate formation in the bovine rumen is related to its slow rate of metabolism by mixed rumen bacteria and its potential as a magnesium chelator (22). Literature values indicate that the stability constant of tricarboxylate for magnesium is 115 (17). Based on this binding capacity, it is conceivable that tricarboxylate could complex a significant portion of dietary magnesium, especially when the magnesium status of the animal is low. Toxicity is obviously related to the relative rates of aconitate conversion to tricarboxylate versus acetate. Experiments are currently being conducted to isolate and characterize rumen bacteria that produce acetate from aconitate.

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LITERATURE CITED


