APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1980, p. 840-846
0099-2240/80/10-0840/07$02.00/0
Vol. 40, No. 4

Characteristics of Anaerobic Oxalate-Degrading Enrichment Cultures from the Rumen

KARL A. DAWSON,[1]*† MILTON J. ALLISON,[1] AND PAUL A. HARTMAN[2]

National Animal Disease Center, Science and Education Administration, Agricultural Research, U.S. Department of Agriculture, Ames, Iowa 50010,[1] and Department of Bacteriology, Iowa State University, Ames, Iowa 50011[2]

Enrichment cultures of rumen bacteria degraded oxalate within 3 to 7 days in a medium containing 10% rumen fluid and an initial level of 45 mM sodium oxalate. This capability was maintained in serially transferred cultures. One mole of methane was produced per 3.8 mol of oxalate degraded. Molecular hydrogen and formate inhibited oxalate degradation but not methanogenesis; benzyl viologen and chloroform inhibited both oxalate degradation and methanogenesis. Attempts to isolate oxalate-degrading bacteria from these cultures were not successful. Oxalate degradation was uncoupled from methane production when enrichments were grown in continuous culture at dilution rates ≥ 0.078 h^{-1}. Growth of the uncoupled population (lacking methanogens) in batch culture was accompanied by degradation of 45 mM oxalate within 24 h and production of 0.93 mol of formate per mol of oxalate degraded. Oxalate degradation by the uncoupled population was not inhibited by molecular hydrogen or formate. Cell yields (grams [dry weight]) per mole of oxalate degraded by the primary enrichment and the uncoupled populations were 1.7 and 1.0, respectively.

Considerable evidence shows that bacteria in the rumen (12, 16, 19, 25, 28) and in the intestinal tracts of other animals (4, 6, 23) degrade oxalate. Anaerobic bacteria appear to be responsible for ruminal oxalate degradation, and the rates of this activity increase in animals adapted to diets containing increasing amounts of oxalate (2). Such adapted animals tolerate diets that contain oxalate at concentrations that are lethal to nonadapted animals (15). The increased rates of oxalate degradation in adapted animals probably reflect selection of oxalate-degrading organisms. This selection may be based upon the ability of the oxalate degraders to use oxalate as a source of energy, but other possibilities cannot be excluded.

Previous attempts to isolate oxalate-degrading anaerobes in pure culture from the rumen have failed (2). The present study with enrichment cultures was conducted because more information about the nature of the oxalate degraders appeared to be a prerequisite for their successful isolation.

MATERIALS AND METHODS

Media. Media for the enrichment and maintenance of oxalate-degrading populations were prepared and inoculated with anaerobic techniques described by Bryant (7). The rumen fluid-oxalate (RFO) enrichment medium contained: Na_2SO_4 (0.2 g), KH_2PO_4 (0.09 g), NaCl (0.09 g), $(NH_4)_2SO_4$ (0.09 g), $MnSO_4 \cdot H_2O$ (0.001 g), $CaSO_4 \cdot 7H_2O$ (0.0001 g), $MgSO_4 \cdot 7H_2O$ (0.0005 g), yeast extract (0.1 g), clarified rumen fluid (10.0 ml), resazurin (0.0001 g), L-cysteine-HCl·H_2O (0.025 g), Na_2CO_3 (0.4 g), and sodium oxalate (0.6 g) in 90 ml of distilled water. This medium was dispensed in 10-ml volumes in culture tubes (16 by 150 mm) under a CO_2 gas phase, unless otherwise indicated. The final pH of the medium was 6.8. For roll tube cultures, RFO medium was prepared with 2% agar and dispensed in 7-ml volumes.

The nonrumen fluid medium used in the chemostat studies contained: K_2HPO_4 (0.0225 g), KH_2PO_4 (0.0225 g), $(NH_4)_2SO_4$ (0.045 g), NaCl (0.045 g), $MgSO_4 \cdot 7H_2O$ (0.0045 g), $CaCl_2 \cdot 6H_2O$ (0.0045 g), resazurin (0.0001 g), Trypticase (BBL Microbiology Systems) (0.2 g), yeast extract (0.05 g), Na_2CO_3 (0.4 g), L-cysteine-HCl·H_2O (0.025 g), sodium oxalate (0.3 g), and a mixture of volatile fatty acids (9) in 100 ml of distilled water. The final pH of this medium was 6.8. The peptone-yeast extract-glucose (PYG) broth (13) was supplemented as needed with 2% agar for use in roll tubes.

Primary enrichment cultures. Inocula for the primary enrichment experiments were obtained from a 1-liter fermentor culture established with rumen contents from a fistulated sheep. The fermentor was maintained by feeding 15 g of ground alfalfa twice daily and by continual infusion of a mineral solution (1) to give a dilution rate of 0.041 h^{-1}. Anaerobic conditions were maintained by flushing with CO_2 when the fermentor was opened for the addition of alfalfa. The culture was adapted by infusion of a sodium oxalate gradient of from 0.031 to 1.56 mmol/h over an 8-day period. Oxalate did not accumulate in the fer-

† Present address: Department of Animal Sciences, University of Kentucky, Lexington, KY 40546.

mentor when the 1.56-mmol/h rate was maintained over a 4-week period. Oxalate degradation rates determined with ^{14}C-labeled oxalate (2) were typically about 4 μmol/ml per h. Experiments with this in vitro fermentor system will be described in a subsequent publication.

Tubes of RFO medium were inoculated with 10-fold dilutions of the fermentor culture (10^{-1} to 10^{-7} ml) prepared in anaerobic dilution solution (8). These primary enrichment cultures were serially transferred in RFO medium when oxalate in the medium could no longer be detected by the qualitative test. All incubations were at 37°C. Absorbance was measured at 600 nm in tubes (16 by 150 mm) with a Bausch & Lomb Spectronic 20 colorimeter.

Chemostat cultures. The chemostat vessels were similar to those described by Siñeriz and Pirt (24). The overflow from the culture vessel was connected with a black rubber tube to an Erlenmeyer flask sealed with a black rubber stopper equipped with a Bunsen valve. Anaerobic conditions were maintained by passing CO_2 through the system while sampling or changing the overflow flask. Media for the chemostats were prepared in aspirator bottles (3 to 5 liter) which were sealed with black rubber stoppers and connected to a butyl rubber balloon. The balloon was filled with CO_2 and maintained a small positive pressure on the reservoir. The flow of media into the culture vessel was controlled with a variable speed peristaltic pump.

Each culture vessel was inoculated with 100 ml of inoculum from a batch culture or from a previously established chemostat. Populations were transferred to sterile culture vessels every 2 weeks to prevent the accumulation of a slime layer on the side of the vessel.

Organic acid determinations. The presence of oxalate in the media was determined by a qualitative calcium precipitation test. For this, 0.1 ml of medium was diluted in 0.9 ml of distilled water, and 0.2 ml of 1% $CaCl_2$ solution was added. The presence of oxalate (concentration greater than about 8 mM) was indicated by development of a milky precipitate within 5 min.

Concentrations of oxalate and other organic acids were measured by gas chromatography of the dibutyl and monobutyl esters (2, 22). Unless stated otherwise, concentrations reported are the means of duplicate samples. Standard deviations were less than 2% of the mean for oxalate concentrations and less than 10% of the mean for formate concentrations. When quantitation of formate was critical, a separate standard curve was established by adding the acid to the test medium.

Gas analysis. In experiments where gas analysis was required, media were prepared in serum bottles that were sealed with black rubber stoppers held by aluminum seals (18). Bottles were inoculated and sampled through the stoppers with disposable hypodermic needles and syringes that had been flushed with CO_2. In hydrogen inhibition studies, H_2 was added to give an initial pressure of 100 mm of mercury above atmospheric pressure. Gas pressures were checked daily and were maintained with a gassing manifold (3) that was modified to allow sampling through a three-way valve. Gas samples were analyzed at room temperature with a model AD-2000 respiratory gas analyzer (Loenco Inc., Altadena, Calif.) modified to adequately separate CO_2 from H_2 by adding 4.6 m of 2.6 mm (inside diameter) Teflon tubing between the first and second detectors. Helium was the carrier gas, and samples were injected with a 0.5-ml injection loop. Quantities of the various gases were estimated by comparing the recorded peak heights with corresponding peaks from standard gas mixtures.

Dry weight determinations. Relationships between optical density and the dry weight of the primary enrichment populations were determined with five 10-ml samples taken after complete oxalate degradation in batch cultures grown on RFO medium. The cells in each sample were collected by centrifugation at 15,000 × *g* in a refrigerated centrifuge and were washed once in 2.0 ml of distilled water. Washed cells were transferred to preweighed beakers by three successive rinses with water. The cells were dried in a 100°C oven for 18 h and placed in a desiccator until a constant weight was reached. Preliminary studies indicated that cell dry weights determined with this technique were not significantly different from those obtained with formaldehyde-fixed cells collected on membrane filters (14).

Dry weight yields per mole of substrate degraded were estimated from measurements of the maximum optical density observed in the test medium and the changes in oxalate concentration (as determined by gas chromatography). Preliminary experiments indicated that yields determined indirectly from the optical density-dry weight relationship were not significantly different from those determined directly from dry weight measurements.

Organisms. *Vibrio succinogenes* was obtained from M. J. Wolin (New York State Department of Health, Albany, New York) and maintained on VSF medium described by Kafkewitz (17). *Desulfovibrio* sp. strain G-11 was obtained from M. P. Bryant (Departments of Dairy Science and Microbiology, University of Illinois, Urbana). This organism was maintained on RFO modified to contain sodium formate (45 mM) instead of sodium oxalate.

RESULTS

Primary enrichment cultures. When RFO medium was inoculated with dilutions containing 10^{-4} ml of fermenter contents, oxalate was degraded to nondetectable concentrations in about 21 days. With 10^{-5} and 10^{-6} ml of inocula, longer incubation was usually required; oxalate was not degraded in RFO medium inoculated with 10^{-7} ml of fermentor contents. On the basis of microscopic examinations, the populations that grew in RFO medium were diverse mixtures of bacteria but were less diverse than the population in the fermentor.

Attempts to develop an enrichment medium more selective than RFO medium included the use of rumen fluid that had been depleted of endogenous substrates by the technique of Dehority and Grubb (11). Evidence was not obtained for: (i) greater selectivity (based on mi-

croscopic examination of the population that grew in the medium); (ii) more rapid growth of the oxalate degraders; or (iii) oxalate degradation with less concentrated inocula from the fermentor. Furthermore, the incubation time for complete oxalate degradation was not shortened by addition of potential cometabolites or electron acceptors, such as succinate, acetate, fumarate, sulfate, or lactate (45 mM each). Oxalate degradation was inhibited by the addition of formate or nitrate (45 mM).

Oxalate-degrading populations were maintained for >1 year by serial transfer in RFO medium and required 3 to 7 days for complete oxalate degradation when a 30% inoculum was used. When smaller inocula were used, longer periods were required for complete oxalate degradation. Although the work reported here deals with enrichments derived from the fermentor, similar enrichments were also obtained in RFO medium inoculated with rumen contents from a sheep adapted to a ration that contained 5% oxalate.

Changes in oxalate concentration in RFO medium and in CH_4 concentration in the gas phase during growth are shown in Fig. 1. Oxalate was degraded after a lag period of about 48 h and was accompanied by accumulation of CH_4 in the gas phase. Oxalate degradation in these cultures occurred only under strictly anaerobic conditions and was not observed when the resazurin indicator become oxidized.

The relationship between oxalate degradation and methane production in RFO medium is shown in Fig. 2. Approximately 3.8 mol of oxalate were degraded for each mole of methane produced. The regression line does not pass through the origin because small amounts of methane were produced in media devoid of added oxalate. Methane production accounted for 95% of the reducing equivalents expected during the oxidation of oxalate to CO_2.

The addition of formate (between 45 and 65 mM) to the RFO medium completely inhibited oxalate degradation, but not methanogenesis. Stoichiometry between methane production and formate degradation was equivalent to that obtained with oxalate (Fig. 3). The oxalate-degrading component of the enrichment was lost in formate-containing medium and was not recovered by subculture in RFO medium. A similar type of inhibition was observed when the gas phase over enrichment cultures in sealed serum bottles contained CO_2 and H_2 (1:1). In these experiments, H_2 utilization was accompanied by methane production. Furthermore, oxalate degradation and methane production by the primary enrichment were completely inhibited by

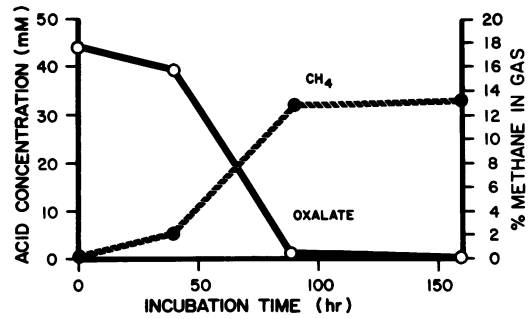

FIG. 1. *Changes in oxalate concentration and methane production during the growth of a primary enrichment population in rumen fluid-oxalate medium; a 10% inoculum was used.*

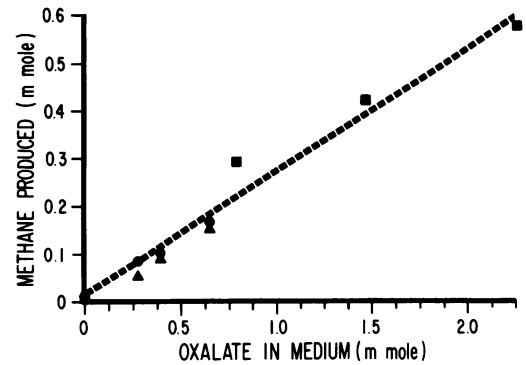

FIG. 2. *The relationship between oxalate degradation and methane production by a primary enrichment in the rumen fluid-oxalate medium containing graded concentrations of oxalate. Experiments 1 (▲) and 2 (●) were conducted in 15-ml volumes in 25-ml serum bottles. Experiment 3 (■) was conducted in 50-ml volumes in 100-ml serum bottles.*

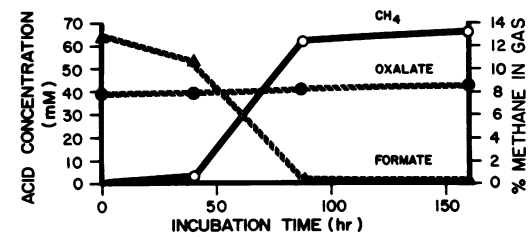

FIG. 3. *The effects of 45 mM formate on oxalate degradation and methane production by a primary enrichment population in rumen fluid-oxalate medium.*

benzyl viologen concentrations greater than 0.52 μM and chloroform concentrations of greater than 1.0 μM (data not shown). Similar low concentrations of these two compounds are known to inhibit methane production in other methanogenic systems (5, 30). Simultaneous inhibi-

tion of methane production and oxalate degradation suggests that these two processes are closely associated in these primary enrichment cultures.

Since methanogenesis appeared to function as a sink for hydrogen generated during oxalate degradation, we postulated that the addition of other hydrogen-utilizing bacteria to the primary enrichment might enhance the enrichment of oxalate degraders and chances for their isolation. Media were inoculated with a 10% inoculum of *Desulfovibrio* G-11 or *V. succinogenes* (which uses H_2 to reduce fumarate to succinate) and with a 1% inoculum of the oxalate enrichment culture. Cultures inoculated with *Desulfovibrio* G-11 degraded all of the oxalate in 7 days, whereas the enrichment culture alone required 10 days. When *V. succinogenes* was similarly used (in RFO medium supplemented with 45 mM sodium fumarate), the time required for complete oxalate degradation was reduced from 10 to 8 days. However, there was no decrease in methane production in these cultures and no increase of succinate production from fumarate. Despite the decrease in time required for oxalate degradation, it did not appear that these organisms could readily compete with the methanogens for hydrogen or dramatically influence the growth of oxalate degraders.

Several attempts were made to use RFO and PYG roll tubes for isolation of oxalate-degrading organisms from the primary enrichments. We picked 30 colonies from several of these roll tubes, but none of the cultures (at least six morphological types) degraded oxalate in RFO medium. In addition, oxalate was not degraded when RFO medium was inoculated with a combination of several different colonies, even when the inoculum was agar from roll tubes inoculated with low dilutions (10^{-2}, 10^{-3} ml) of the primary enrichment culture.

Enrichment in continuous culture. A methanogenic, oxalate-degrading enrichment was established in a chemostat that was fed RFO medium modified to contain 22.5 mM of sodium oxalate. The populations in the chemostats were maintained for several weeks at dilution rates of $<0.078\ h^{-1}$ without accumulation of oxalate. Typically, the gas phase over these cultures contained about 8% methane. No formate or hydrogen was detected. When the dilution rate in chemostat cultures was increased to 0.078 h^{-1}, growth was accompanied by the accumulation of formate and a slight increase in the concentration of residual oxalate (Fig. 4); neither methane nor hydrogen was detected in the gas phase over the culture. Because oxalate degradation by the population that developed at the increased dilution rate was not accompanied by (or coupled to) methanogenesis, we have called this new population the uncoupled population. The infusion of the nonrumen fluid medium instead of modified RFO medium did not significantly alter the fermentation pattern observed in the chemostat or cause an increase in the oxalate concentration (Fig. 4). Although there was a slight decrease in the amount of formate produced, there was no detectable increase in other reduced end products (i.e., hydrogen, methane, propionate, and succinate) in the chemostat.

In other experiments (data not shown), the oxalate-degrading population was maintained at dilution rates of up to 0.41 h^{-1}, a rate that requires a generation time of about 2.5 h. The methanogenic population was maintained only

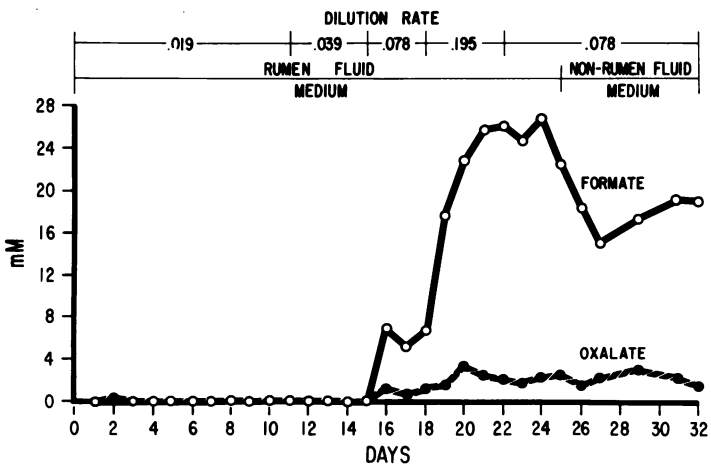

FIG. 4. *Changes in oxalate and formate concentrations during growth of an oxalate-degrading population in an anaerobic chemostat culture at several dilution rates.*

at dilution rates of less than 0.78 h^{-1}. These observations suggest that the methanogenic portion of the population had a minimum generation time of about 12.8 h under these culture conditions.

The physiological properties and growth characteristics of the uncoupled population were significantly different from those of the methanogenic primary enrichment. When the uncoupled population was serially transferred on the RFO medium, no methane was produced, even after prolonged incubation, and approximately 0.93 mol of formate was produced for each mole of oxalate degraded. Although both the primary enrichment and the uncoupled population degraded oxalate only under anaerobic conditions, the uncoupled population required much less time for complete degradation (<24 h) and required a much smaller inoculum for maintenance (<1%). Oxalate degradation by the uncoupled population was not inhibited by H_2 (1:1 in the gas phase), formate (45 mM), or benzyl viologen (5.2 μM). The uncoupled population was a complex mixture of bacteria that could not easily be distinguished from the primary enrichment cultures by microscopic examination of stained or wet mount preparations.

Cell yields. The amount of growth and the time required to reach maximum growth in the primary enrichment cultures were related to the amount of oxalate in RFO medium (Fig. 5). In most experiments, oxalate was degraded after 1 to 3 days of incubation and was complete at the time absorbance was at a maximum. Dry weight measurements on five samples taken from these growing cultures indicated that there were 0.61 ± 0.24 g (dry weight)/ml per optical density unit (mean ± standard deviation). A cell yield (Y_{ox}) at the maximum optical density of 1.68 ± 0.24 g (dry weight)/mol of oxalate (n = 3) was calculated from this relationship. Growth in the medium without oxalate was subtracted from that in the medium with oxalate to determine the yield values given here.

Growth of the uncoupled population was also related to the oxalate concentration in the medium (Fig. 6). A yield of 1.02 ± 0.23 g (dry weight)/mol of oxalate (n = 3) was obtained, assuming that cell dry weight per optical density unit was the same as that in the primary enrichments. This yield was 60% of that in the primary enrichment culture.

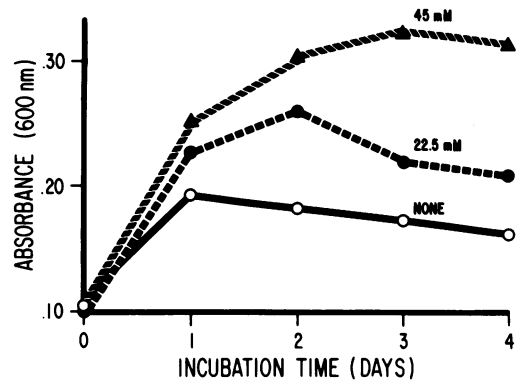

FIG. 5. *Growth responses of a primary enrichment population during growth in rumen fluid-oxalate medium containing different concentrations of oxalate.*

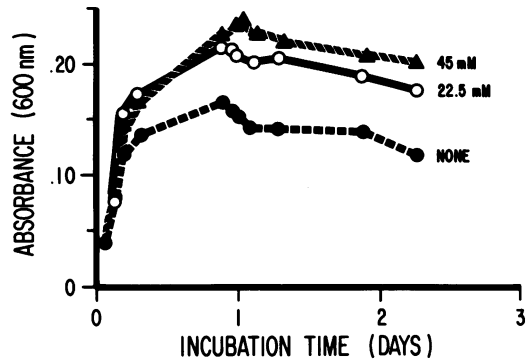

FIG. 6. *Growth responses of an uncoupled oxalate-degrading population during growth in rumen fluid-oxalate medium containing different concentrations of oxalate.*

DISCUSSION

The RFO enrichment medium used in this study permitted the maintenance of anaerobic oxalate-degrading populations that were less complex than those in the fermentor cultures. These primary enrichments, however, were still complex mixtures of bacteria. Many of the organisms in these enrichments were apparently not directly responsible for oxalate degradation because these organisms grew in the enrichment medium in the absence of oxalate, as well as when the ability to degrade was lost in formate-containing media. Attempts to markedly decrease the time required for oxalate degradation and to increase the selectivity of the medium by using deletion procedures, by adding other potential substrates, cometabolites, and electron acceptors, or by coculture with hydrogen-using bacteria were not successful.

The degradation of oxalate in the primary enrichment cultures could represent a short food chain, with methanogenesis serving as the final electron sink. This process was not carried out by only one organism because methanogenesis was separated from oxalate degradation in chemostat cultures at high dilution rates. In-

stead, oxalate degradation and accompanying methane production in these enrichments were the results of the action of at least two organisms, i.e., an oxalate degrader and a methanogen. Results of studies with benzyl viologen and chloroform show that the oxalate-degrading component of the primary enrichments was closely associated with the methanogenic component of the population. The methanogens were thought to be important in removing the inhibitory effects of formate or hydrogen or both. A number of similar microbial interactions have been described (29). The relationship between oxalate degradation and methanogenesis, however, was not obligatory because oxalate degradation by the non-methanogenic, uncoupled population was not inhibited by formate, hydrogen, or by inhibitors of methanogenesis. In RFO medium, other hydrogen-utilizing bacteria (*V. succinogenes* and *Desulfovibrio* G-11) did not successfully compete with the methanogens for hydrogen and only slightly stimulated oxalate degradation in the primary enrichments. Interactions between the oxalate degraders and methanogens, thus, are not yet fully explained and may involve other organisms in the primary enrichment populations.

Growth of both the primary enrichment and the uncoupled populations was related to the concentration of oxalate in the enrichment medium. This relationship suggests that some part of each of these oxalate-degrading populations used oxalate as a primary energy source. The cell yield of the methanogenic population was greater than that of the uncoupled population (Table 1). The difference was expected in view of differences in the end products produced in each of these cultures and the free energy changes predicted from their formation. The methanogenic, primary enrichment produced methane (and presumably CO_2) as major end products and produced cell yields of about 1.7 g (dry weight)/mol. The growth of the methanogens would account for the difference observed between the Y_{ox} of the primary enrichment and the Y_{ox} of the uncoupled population. This difference (0.7 g [dry weight]/mol) probably resulted from the use of formate by the methanogenic component and approximates the yields reported when other methanogenic bacteria were grown on hydrogen (20).

TABLE 1. *Free energy changes and cell yields for anaerobic oxalate degradation by enrichment cultures*

Enrichment culture	$\Delta G^{\circ\prime}$[a] (kJ/reaction)	$\Delta G^{\circ\prime}$/oxalate (kJ/mol)	Y_{ox}[b] (g [dry wt]/mol)
Primary enrichment ($4\ oxalate^- + 5\ H_2O \rightarrow 3H^+ + CH_4 + 7HCO_3^-$)	−298.9	−74.8	1.7
Uncoupled population ($oxalate^- + H_2O \rightarrow HCO_3^- + H^+ + formate^-$)	−42.2	−42.2	1.0

[a] Free energy changes were calculated from the data given by Thauer et al. (26).

[b] Yields per mole of oxalate were determined as indicated in the text.

The cell yields agree well with theoretical free energy change estimates for the overall reactions presumed to occur in the coupled and uncoupled populations (Table 1). Both yields and theoretical free energy change in the uncoupled population were about 57% of those in the methanogenic primary enrichment. These data suggest that the oxalate degraders in these populations used the free energy released during oxalate degradation as efficiently as the methanogen used the energy released during the degradation of formate (produced during oxalate degradation) in the primary enrichments.

The successful selection and maintenance of enriched, anaerobic, oxalate-degrading populations from mixed populations of rumen bacteria confirm the results of earlier studies that suggested that oxalate degradation in the rumen is a bacterial process. The data from this investigation also suggest that the ability to use oxalate as an energy-yielding substrate is the basis for the selection of oxalate-degrading populations in the rumen of oxalate-adapted animals. Many of the plants ingested by ruminants contain measurable quantities of oxalate (21, 27). Repeated intakes of low concentrations of oxalate could allow oxalate-using bacteria to maintain themselves in low numbers in the rumen and account for the low, but measurable, rates of oxalate degradation in the rumen of animals on an alfalfa diet (2). The survival of oxalate degraders in the rumen of such animals means that adaptation to oxalate-containing feeds does not require the introduction of new organisms in the feed, as suggested by Dodson (12). Increased rates of ruminal oxalate degradation in adapted animals (2) apparently depends on selection of anaerobic organisms that are already present in the rumen.

Attempts to isolate oxalate degraders from adapted animals (2) and from primary enrichment cultures may have been unsuccessful because of the relatively low numbers of oxalate degraders in these populations. The uncoupled population was physiologically simpler than the enriched population and contained sufficient

numbers of oxalate degraders to permit the development of oxalate-degrading colonies on roll tube media. Isolation and characterization of a pure culture of an oxalate-degrading, obligate anaerobe from this uncoupled population are described in a companion paper (10).

LITERATURE CITED

1. **Abe, M., and F. Kumeno.** 1973. *In vitro* simulation of rumen fermentation: apparatus and effects of dilution rate and continuous dialysis on fermentation and protozoal population. J. Anim. Sci. **36**:941-948.
2. **Allison, M. J., E. T. Littledike, and L. F. James.** 1977. Changes in ruminal oxalate degradation rates associated with adaptation to oxalate ingestion. J. Anim. Sci. **45**:1173-1179.
3. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781-791.
4. **Barber, H. H., and E. J. Gallimore.** 1940. The metabolism of oxalic acid in the animal body. Biochem. J. **34**: 144-148.
5. **Bauchop, T.** 1967. Inhibition of rumen methanogenesis by methane analogues. J. Bacteriol. **94**:171-175.
6. **Brune, H., and H. Bredehorn.** 1961. On the physiology of bacterial degradation of calcium oxalate and the ability to utilize calcium from calcium oxalate in the pig. Z. Tierphysiol. Tierernaehr. Futtermittelkd. **16**: 214-236.
7. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324-1328.
8. **Bryant, M. P., and L. A. Burkey.** 1953. Cultural methods and some characteristics of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205-217.
9. **Caldwell, D. R., and M. P. Bryant.** 1966. Medium without rumen fluid for the nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. **14**: 794-801.
10. **Dawson, K. A., M. J. Allison, and P. A. Hartman.** 1980. Isolation and some characteristics of anaerobic oxalate-degrading bacteria from the rumen. Appl. Environ. Microbiol. **40**:833-839.
11. **Dehority, B. A., and J. A. Grubb.** 1976. Basal medium for the selective enumeration of rumen bacteria utilizing specific energy sources. Appl. Environ. Microbiol. **32**: 703-710.
12. **Dodson, M. E.** 1959. Oxalate ingestion studies in the sheep. Aust. Vet. J. **35**:225-233.
13. **Holdeman, L. V., E. P. Cato, and W. E. C. Moore (ed.).** 1977. Anaerobe laboratory manual, 4th ed. Virginia Polytechnic Institute and State University, Blacksburg.
14. **Isaacson, H. R., F. C. Hinds, M. P. Bryant, and F. N. Owens.** 1975. Efficiency of energy utilization by mixed rumen bacteria in continuous culture. J. Dairy Sci. **58**: 1645-1659.
15. **James, L. F., and J. E. Butcher.** 1972. Halogeton poisoning of sheep: effect of high level oxalate intake. J. Anim. Sci. **35**:1233-1238.
16. **James, L. F., J. C. Street, and J. E. Butcher.** 1967. *In vitro* degradation of oxalate and of cellulose by rumen ingesta from sheep fed *Halogeton glomeratus*. J. Anim. Sci. **26**:1438-1444.
17. **Kafkewitz, D.** 1975. Improved growth media for *Vibrio succinogenes*. Appl. Microbiol. **29**:121-122.
18. **Miller, T. L., and M. J. Wolin.** 1974. A serum bottle modification of the Hungate technique for cultivating obligate anaerobes. Appl. Microbiol. **27**:985-987.
19. **Morris, M. P., and J. Garcia-Rivera.** 1955. The destruction of oxalates by rumen contents of cows. J. Dairy Sci. **38**:1169.
20. **Robertson, A. M., and R. S. Wolfe.** 1970. Adenosine triphosphate pools in *Methanobacterium*. J. Bacteriol. **102**:43-51.
21. **Rumsey, T. S., C. H. Noller, C. L. Rhykerd, and J. C. Burns.** 1967. Measurement of certain metabolic organic acids in forage, silage and rumen fluid by gas chromatography. J. Dairy Sci. **50**:214-219.
22. **Salanitro, J. P., and P. A. Muirhead.** 1975. Quantitative method for gas chromatographic analysis of short-chain monocarboxylic and dicarboxylic acids in fermentation media. Appl. Microbiol. **29**:374-381.
23. **Shirley, E. K., and K. Schmidt-Nielsen.** 1967. Oxalate metabolism in the pack rat, sand rat, hamster and white rat. J. Nutr. **91**:496-502.
24. **Siñeriz, F., and S. J. Pirt.** 1977. Methane production from glucose by a mixed culture of bacteria in the chemostat: the role of *Citrobacter*. J. Gen. Microbiol. **101**:57-64.
25. **Talapatra, S. K., S. C. Ray, and K. C. Sen.** 1948. Calcium assimilation in ruminants on oxalate-rich diet. J. Agric. Sci. **38**:163-173.
26. **Thauer, R. K., K. Jungermann, and K. Decker.** 1977. Energy conservation in chemotrophic anaerobic bacteria. Bacteriol. Rev. **41**:100-180.
27. **Ward, G., L. H. Harbers, and J. J. Blaha.** 1979. Calcium-containing crystals in alfalfa. Their fate in cattle. J. Dairy Sci. **62**:715-722.
28. **Watts, P. S.** 1957. Decomposition of oxalic acid *in vitro* by rumen contents. Aust. J. Agric. Res. **8**:266-270.
29. **Wolin, M. J.** 1974. Metabolic interaction among intestinal microorganisms. Am. J. Clin. Nutr. **27**:1320-1328.
30. **Wolin, E. A., R. S. Wolfe, and M. J. Wolin.** 1964. Viologen dye inhibition of methane formation by *Methanobacillus omelianskii*. J. Bacteriol. **87**:993-998.