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Assessment of Reductive Acetogenesis with Indigenous Ruminal Bacterium Populations and *Acetitomaculum ruminis*

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The objective of this study was to evaluate the role of reductive acetogenesis as an alternative H_2 disposal mechanism in the rumen. H_2/CO_2-supported acetogenic ruminal bacteria were enumerated by using a selective inhibitor of methanogenesis, 2-bromoethanesulfonic acid (BES). Acetogenic bacteria ranged in density from 2.5×10^5 cells/ml in beef cows fed a high-forage diet to 75 cells/ml in finishing steers fed a high-grain diet. Negligible endogenous acetogenic activity was demonstrated in incubations containing ruminal contents, $NaH^{13}CO_3$, and 100% H_2 gas phase since [U-^{13}C]acetate, as measured by mass spectroscopy, did not accumulate. Enhancement of acetogenesis was observed in these incubations when methanogenesis was inhibited by BES and/or by the addition of an axenic culture of the rumen acetogen *Acetitomaculum ruminis* 190A4 (10^7 CFU/ml). To assess the relative importance of population density and/or H_2 concentration for reductive acetogenesis in ruminal contents, incubations as described above were performed under a 100% N_2 gas phase. Both selective inhibition of methanogenesis and *A. ruminis* 190A4 fortification (>10^5 CFU/ml) were necessary for the detection of reductive acetogenesis under H_2-limiting conditions. Under these conditions, H_2 accumulated to 4,800 ppm. In contrast, H_2 accumulated to 400 ppm in incubations with active methanogenesis (without BES). These H_2 concentrations correlated well with the pure culture H_2 threshold concentrations determined for *A. ruminis* 190A4 (3,830 ppm) and the ruminal methanogen 10-16B (126 ppm). The data demonstrate that ruminal methanogenic bacteria limited reductive acetogenesis by lowering the H_2 partial pressure below the level necessary for H_2 utilization by *A. ruminis* 190A4.

In the rumen, methanogenic bacteria utilize hydrogen (H_2) to reduce carbon dioxide (CO_2) and/or formate to methane (CH_4) as follows: $CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$. Eructation of methane constitutes a 3 to 12% loss of gross energy intake for ruminants (4, 5, 17, 27) and contributes to atmospheric methane concentrations implicated in global warming (24). Johnson and Johnson (29) recently estimated that beef cattle account for 67% of methane emissions by the U.S. cattle herd. Beef cows account for 21% of the herd and 40% of the herd's methane emissions. Feedlot cattle are principally steers and account for 15% of the herd and 6% of methane emissions. These two classes of cattle are widely divergent in their dietary management and tractability for fermentative modification. Previous efforts to minimize digestible energy loss by suppressing ruminal methanogenesis have included the use of antibiotics, ionophores, or halogenated methane analogs (15, 43). Short-duration experiments with these analogs succeeded in suppressing CH_4 production, but H_2 and formate accumulated (16) and food consumption by sheep was adversely affected (13). The accumulation of H_2 indicated that halogenated CH_4 analogs disrupted interspecies H_2 transfer and thus were not sufficiently selective in suppression of methanogenesis. As a consequence, it is now realized that minimization of ruminal methane production needs to involve a strategy whereby electron disposal via interspecies H_2 transfer is not disrupted, and it would be advantageous if reducing equivalents were deposited in a metabolite(s) which serves as a substrate for ruminant tissue metabolism. One novel approach is the involvement of reductive acetogenesis. Reductive acetogenic bacteria reduce 2 mol of CO_2 to acetate by oxidation of H_2 as follows: $2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$. Diversion of energy from eructated CH_4 to acetate by H_2/CO_2-consuming acetogenic bacteria could potentially enhance the energetic efficiency of ruminants and decrease methane emissions (34).

The potential importance of H_2/CO_2-utilizing acetogenic bacteria in the rumen has been described; however, their capacity to effect a total synthesis of acetate from H_2 and CO_2 in ruminal contents and the factors influencing the magnitude of this activity have not been investigated. The presence of H_2/CO_2-utilizing acetogenic bacteria in the rumen has been shown (22, 25). *Acetitomaculum ruminis* produces acetate via heterotrophic growth on, for example, glucose and ferulic acid and via autotrophic growth on formate, carbon monoxide (CO), and H_2/CO_2 (30). Acetate produced via autotrophic growth would constitute a competition with methanogenesis for hydrogen. While reductive acetogenesis is quantitatively important in the termite hindgut (6), there are apparently no reports of nonmethanogenic ruminants. On the basis of data for nonruminal isolates, it has been hypothesized that ruminal acetogenic bacteria are not able to compete with ruminal methanogenic bacteria due to a less effective H_2-scavenging ability, yet there is a paucity of evidence to support this hypothesis.

Recent efforts to study ruminal acetogens and acetogenesis have hinged on the use of 2-bromoethanesulfonic acid (BES), an analog for coenzyme M (21). This coenzyme is essential for the growth of some of the ruminal *Methanobrevibacter* species

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(35). Whereas chloroform was a nonselective inhibitor of methanogenesis and other metabolisms dependent on transmethylation (23), BES has been used as a selective inhibitor of ruminal methanogenic bacteria because it is a methylreductase inhibitor (44).

In this study, a series of experiments were conducted to assess the presence, endogenous activity, and competitiveness of reductive acetogenesis in the bovine rumen. When cattle were the source of inocula, their nutritional management was intended to simulate the beef cow and feedlot sectors of the U.S. beef cattle industry. The principal energy source in beef cow diets is forage (10), whereas feedlot cattle are fed a high-grain diet containing an ionophore (11). Specifically, the population size of reductive acetogenic bacteria was determined for cattle in these two scenarios, and endogenous reductive acetogenic activity was quantified in rumen-like incubations by using mass spectroscopy (MS). We also investigated the ability of *A. ruminis* 190A4 to compete with rumen methanogens by altering the concentration of this acetogen or H_2 in ruminal contents and measurement of its threshold for H_2.

MATERIALS AND METHODS

Media and growth conditions. For enumeration studies, AC-11 medium, used for the cultivation of acetogenic bacteria from the termite hindgut (6), was modified for the cultivation of acetogenic bacteria from the rumen. AC-B1 contained (per liter) the following: KH_2PO_4, 0.28 g; K_2HPO_4, 0.94 g; NaCl, 0.14 g; KCl, 0.16 g; $MgSO_4 \cdot 7H_2O$, 0.02 g; NH_4Cl, 0.5 g; $CaCl_2 \cdot 2H_2O$, 0.001 g; trace mineral solution (25), 10 ml; vitamin solution (25), 10 ml; yeast extract, 0.5 g; $NaHCO_3$, 6.0 g; reducing agent (2.5% [wt/vol] each cysteine hydrochloride $\cdot H_2O$ and $Na_2S \cdot 9H_2O$, pH 10.0), 10 ml; clarified bovine rumen fluid, 100 ml; BES (sodium salt; filter sterilized) as indicated, 20 ml; and resazurin, 0.001 g. Broth media were boiled and cooled under a flow of 80% N_2–20% CO_2 (vol%) prior to addition of reducing agent and $NaHCO_3$. The final volume per tube was 5 ml. The final pH of the medium was approximately 6.8, with a headspace gas of 304 kPa of 80% N_2–20% CO_2 (vol%). Serum tubes were incubated horizontally with 304 kPa of 80% H_2–20% CO_2 (vol%) or 80% N_2–20% CO_2 (vol%) at 39°C and 200 rpm.

For pure-culture H_2 threshold studies, *A. ruminis* strains (provided by the Upjohn Co., Kalamazoo, Mich.) (25) and *Acetobacterium woodii* (ATCC 29683) were grown on BSW-1 medium. BSW-1 medium contained (per liter) the following: KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; KCl, 0.5 g; NaCl, 7.0 g; Na_2SO_4, 0.1 g; $MgCl_2 \cdot 6H_2O$, 1.2 g; $CaCl_2 \cdot 2H_2O$, 0.15 g; yeast extract, 1.5 g; resazurin, 0.001 g; trace mineral solution (25), 10.0 ml; and vitamin solution (25). The medium was boiled and cooled under a flow of 80% N_2–20% CO_2 (vol%), and then 6.0 g of $NaHCO_3$ and 10.0 ml of reducing agent (2.5% [wt/vol] each cysteine hydrochloride $\cdot H_2O$ and $Na_2S \cdot 9H_2O$, pH 10) were added. Final pH of this medium was 7.4. Ruminal methanogen 10-16B (32) was grown in pure culture on M1 medium, which contained (per liter) the following: minerals 1 and 2 (9), 25 ml of each; resazurin, 0.001 g; yeast extract, 2.0 g; Trypticase, 2.0 g; sodium acetate, 2.5 g; Tween 80, 0.0125 g; volatile fatty acid (VFA) solution (12), 5.0 ml; vitamin solution (25), 10.0 ml; and trace mineral solution (25), 10.0 ml. Final preparations were done as described for AC-B1 but under a flow of 100% CO_2 and resulted in a medium with pH 7.0. *Sporomusa termitida* was provided by J. A. Breznak (Michigan State University, East Lansing) and was grown on AC-20 medium (7). Microorganisms for pure-culture H_2 threshold studies were incubated horizontally with 304 kPa of 80% H_2–20% CO_2 (vol%) at 200 rpm. *A. ruminis* and methanogen 10-16B were grown at 38°C. *S. termitida* and *A. woodii* were grown at 30°C.

A. ruminis 190A4 was enumerated on AC-B1 agar plates (without BES). Total viable anaerobes were enumerated on AC-B1 agar plates (without BES) supplemented with 0.05% (wt/vol) each soluble starch, cellobiose, and glucose. Agar plates were incubated at 39°C in a 2.5-gal paint can (25, 28) for 14 days with 304 kPa of 80% H_2–20% CO_2 (vol%). Rumen methanogenic bacteria were enumerated by the three-tube most-probable-number (MPN) technique on AC-B1 medium (without BES) supplemented with 2 g of Trypticase per liter. Serum tubes were incubated horizontally with 304 kPa of 80% H_2–20% CO_2 (vol%) at 39°C and 200 rpm.

Animal diets and rumen samples. For enumeration studies, a rumen sample was obtained via stomach tube 1.5 h postfeeding from each of four beef steers fed once daily a typical finishing, i.e., high-grain (90 corn and supplement:10 corn silage [dry matter basis]) diet containing 0.03 g of monensin/kg of dry matter. The average rumen sample pH for animals on the high-grain diet was 6.2 ± 0.2 (standard error of the mean [SEM]). A high-forage rumen sample was obtained 2 to 4 h postfeeding from each of four beef cows fed alfalfa-grass hay once daily. Average rumen sample pH for animals on the high-forage diet was 7.1 ± 0.1 (SEM). For in vitro competition studies, beef cows described above were subsequently fistulated and fed alfalfa-grass hay. A ventral rumen sample from each animal was obtained 2 h postfeeding and passed through a 2-mm-mesh screen in an anaerobic glove box. Samples were pooled across three cattle to incorporate animal variation into the rumen sample. The pooled rumen sample pH was adjusted to 6.1 since addition of $NaH^{13}CO_3$ for in vitro incubations excessively increased the pH of unadjusted samples.

Enrichment and enumeration studies. H_2/CO_2-supported acetogenic bacteria were enumerated by the three-tube MPN method. Rumen fluid was transferred to an anaerobic chamber (gas phase, 78% N_2–17% CO_2–5% H_2 [vol%]) for preparation of serial dilutions. Six tubes of AC-B1 medium with 2.5 mM BES were inoculated with each dilution. The tubes were evacuated and pressurized to 304 kPa with a gassing manifold (1), three tubes with 80% H_2–20% CO_2 (vol%) and three with 80% N_2–20% CO_2 (vol%) for controls. Tubes were incubated for 8 to 12 days at 39°C and 200 rpm. Optical density of the enrichment cultures was monitored every other day, with periodic repressurization with the appropriate gas mixtures. H_2/CO_2-incubated cultures were considered positive for acetogenic bacteria when the optical density at 600 nm was ≥0.3 over that in the N_2/CO_2-incubated tubes, methane was not detected in the headspace gas, and the pH was ≤6.1.

Aliquots from each H_2/CO_2-incubated tube which showed no growth were transferred to duplicate tubes of fresh AC-B1 medium. One tube was pressurized with 80% H_2–20% CO_2 (vol%), the other tube was pressurized with 80% N_2–20% CO_2 (vol%), and both were incubated in the presence of BES as described for an additional 8 to 12 days. A third and final transfer was done for the remaining H_2/CO_2-incubated tubes, which showed no growth. This incubation protocol for the enumeration of acetogenic bacteria was adopted since an 8- to 12-day incubation for each of three transfers resulted in maximum enrichment of acetogenic bacteria (31a). The MPN was calculated from the tables of deMan (19). Our detection limit for the MPN was calculated to be less than 15 cells/ml (31a). Positive MPN tubes were confirmed to be acetogenic when acetate concentrations were significantly greater in the H_2/CO_2-incubated tubes than in the control tubes.

$^{13}CO_2$ fixation studies. Serum vials (70 ml) containing 60 mM potassium phosphate buffer were maintained in an anaerobic chamber (gas phase, 78% N_2–17% CO_2–5% H_2 [vol%]) for at least 1 h prior to autoclaving. After cooling, sterile BES (5.0 mM, final concentration; sparged with 100% N_2) was added to vials in the anaerobic chamber followed by 9 ml of a freshly strained, pooled rumen sample. Vials were evacuated for 5 min while being shaken at 200 rpm and were then pressurized with 124 kPa of 100% H_2 or 100 kPa of 100% N_2 with a gassing manifold (1). Residual CO_2 remaining after evacuation was less than 0.01 mmol as measured by the $BaCO_3$ method (31). Gas volumes were measured with a pressure transducer (model PX126-015DV; Omega Engineering, Stamford, Conn.) (20). The volume of gas was calculated from the measured internal pressure and with reference to a standard curve. Initial H_2 concentration in the headspace gas was approximately 6.0 mmol per vial. $NaH^{13}CO_3$ solution (75 mM [final concentration] as determined by the $BaCO_3$ method [31]) was added to all vials followed immediately by addition of an *A. ruminis* 190A4 inoculum as indicated. $NaH^{13}CO_3$ solution was prepared by dissolving $NaH^{13}CO_3$ (greater than 98% ^{13}C enriched; Isotec Inc., Miamisburg, Ohio) in sterile CO_2-free water containing 1% (vol/vol) reducing agent (described above). The final volume in the vials was 10.7 ml, and the initial and final pH of the reaction mixture were approximately 7.1 and 6.9, respectively. Vials were incubated at 39°C with shaking at 200 rpm for approximately 48 h. Reactions were terminated by addition of 0.4 ml of concentrated HCl. After mixing and allowing CO_2 equilibration for 15 min, final gas volumes were measured. Headspace gas and liquid phase were sampled for gas chromatographic (GC), liquid chromatographic, and GC-mass spectometry (MS) analyses.

For experiments simulating physiological H_2 concentrations in the rumen, vials were prepared as described above, with the following exceptions: vials contained 80 mM potassium phosphate buffer and 40 mM 3-[*N*-morpholino]-2-hydroxypropanesulfonic acid (MOPSO; sodium salt) for increased buffering capacity; 0.38 g of alfalfa (ground through a 2-mm-mesh screen) was added as a slowly degradable carbohydrate and a source of H_2; and vials were evacuated and pressurized with 6.9 kPa of 100% N_2. Initial and final pHs after 48 h of incubation were approximately 7.3 and 6.6, respectively. MOPSO was found to have no effect on H_2/CO_2-consuming methanogenic activity in ruminal contents and *A. ruminis* 190A4 acetogenic activity (31a).

Pure-culture H_2 threshold studies. Bacteria were cultured in 125-ml serum bottles each containing 25 ml of medium under 232 kPa of 80% H_2–20% CO_2 (vol%). After incubation, the bottles were flushed with sterile 80% N_2–20% CO_2 (vol%) and evacuated three times, and the contents were pooled. To the pooled cultures, an equal volume of sterile medium was added under a flow of 80% N_2–20% CO_2 (vol%). Aliquots of 5 ml of suspension were distributed into sterile tubes under a flow of 80% N_2–20% CO_2 (vol%). Tubes were sealed with butyl rubber stoppers held in place with aluminum seals. Ten milliliters of 80% N_2–20% CO_2 (vol%) was added to some of the tubes to serve as controls for endogenous H_2 production. Four of these tubes immediately were analyzed for H_2 so that the initial background concentration of H_2 could be estimated. Finally, 10 ml of 1.2% H_2–75.2% N_2–23.6% CO_2 (vol%) was added to another set of tubes to provide 6,000 ppm of H_2 as the substrate for estimation of the H_2 thresholds. The initial H_2 concentration was in excess of the H_2 threshold values measured for all selected microorganisms. Additional tubes of medium only

(uninoculated) were prepared as described above to serve as controls for abiological consumption and production of H_2.

All tubes except those used for immediate H_2 analysis were incubated for 5 to 7 days with constant agitation. Initial observations revealed that the threshold was approached after 2 days of incubation. We allowed 5 to 7 days of incubation to ensure enough time for the threshold concentration to be reached. Subsequently, headspace gas pressure in tubes was measured with a pressure transducer (model PX102-006 GV; Omega Engineering), and the concentration of H_2 remaining in the headspace gas was determined with a mercury reduction detector as described below.

Liquid and gas chromatographic analyses. The concentration of VFAs (formate, acetate, propionate, and butyrate) was measured by liquid chromatography. Samples were prepared and analyzed as described by Barlaz et al. (2). The gas phase of the enrichment cultures and in vitro incubations was analyzed for H_2, CH_4, and CO_2 by GC by injecting the sample into a Packard 438 gas chromatograph (Chrompack, Raritan, N.J.) equipped with a model 914 thermal conductivity detector and HP 3390A integrator (Hewlett-Packard, Avondale, Pa.) for data acquisition. The column used was 120/140 Carbosieve S2 (2.7 m by 3.2 mm [outside diameter]; Supelco, Bellefonte, Pa.). Operating conditions for the gas chromatograph were as follows: N_2 carrier gas, 30 ml/min; column temperature, 200°C; injector and detector temperature, 210°C; and injection volume, 0.4 ml.

For pure-culture H_2 threshold studies, H_2 was measured with a mercury reduction detector (Trace Analytical, Menlo Park, Calif.) and expressed as parts per million. Headspace gas was vented through a 20-μl sample loop and injected onto an 80/100-mesh molecular sieve 5A chromatography column (3.2 mm by 1.8 m). Chromatographic conditions were as follows: column temperature, 50°C; carrier gas, chromatographic-grade helium with flow rates of 60 ml/min for *A. ruminis* strains, 20 ml/min for the methanogen, and 40 ml/min for *S. termitida* and *A. woodii*. The H_2 concentration in a tube was calculated after adjustment for the gas pressure as follows:

$$H_2 \text{ threshold (ppm)} = \left[\frac{\text{ppm } H_2 \times (22.2 \text{ ml} + \text{ml of gas vented})}{22.2 \text{ ml}}\right]$$

where 22.2 ml is the gas-phase volume of the tube. The detection limit was dependent on flow rate; therefore, the detection limits were <1 ppm for methanogens and <10 ppm for the acetogens.

MS analysis. Mass spectra of VFAs were determined by GC-MS to calculate the ratio of ^{13}C- to ^{12}C-labeled acetate, propionate, and butyrate. Samples for GC-MS were derivatized to butyl esters and extracted from an aqueous phase into hexane as described by Salanitro and Muirhead (40). The mass spectrum fragmentation pattern for butyl acetate compared well with published spectra and the fragment ion peak for [U-^{12}C]acetate. Derivatization yielded an average 98.5% recovery for VFA compounds. GC-MS samples were carried onto a DB-1 column (0.25 mm by 12 m; J&W Scientific, Folsom, Calif.) fitted in an HP 5890 gas chromatograph operating in split mode (1:50). Helium was the carrier gas, with a mean linear velocity of 0.8 ml/min. The temperature was held at 40°C for 2 min, programmed to increase to 70°C at 5°C/min, then incremented to a final temperature of 120°C at 20°C/min and held at that temperature for 1 min, for a total run time of 11.5 min. Eluting compounds were volatilized into an HP 5970 mass selective detector (70-eV ionization) controlled by an HP UNIX data station, and total ion chromatograms were reconstructed. From abundance ratios of fragment ions, e.g., $-O^{12}C^{12}CH_3$ (mass = 43), $-O^{13}C^{12}CH_3$ or $-O^{12}C^{13}CH_3$ (mass = 44), and $-O^{13}C^{13}CH_3$ (mass = 45) for butyl acetate species, and quantitation of total VFAs by liquid chromatography, the concentration of a ^{13}C isotope was calculated. Recovery of [U-^{13}C]acetate was 101.0%, which indicated that the method was sufficiently accurate to use in quantitative fermentation studies.

RESULTS

Enumeration of H_2/CO_2-supported acetogenic bacteria from the rumen. H_2/CO_2-supported acetogenic bacteria in the rumen were enumerated by using a selective inhibitor of methanogenesis, BES. For cattle receiving a high-forage diet, H_2/CO_2-supported acetogenic bacteria ranged in population density from 3.5×10^1 to 2.6×10^5 cells/ml of rumen fluid (Fig. 1A). The total viable anaerobe concentration was between 1.5×10^8 and 4.8×10^8 CFU/ml of rumen fluid for all high-forage enrichments. For cattle receiving a typical finishing (high-grain) diet, H_2/CO_2-supported acetogenic bacteria were less numerous ($P < 0.05$) than for the high-forage diet and ranged in population density from 2 to 75 cells/ml of rumen fluid (Fig. 1B). Total viable anaerobic population for this diet was 1.4×10^9 to 4.7×10^9 CFU/ml of rumen fluid. Additional enrichments from the high-grain diet rumen samples were done on AC-B1 medium at pH 6.1. The medium at pH 6.1 was expected to be more habitat simulating (for high-grain diet) than the medium at pH 6.8; however, no H_2/CO_2-supported acetogenic bacteria were detected (data not shown). H_2/CO_2-supported acetogenic cultures were confirmed to be acetogenic. Acetate accumulated in the H_2/CO_2-incubated tubes (58.6 mM ± 6.3) over that in the N_2/CO_2-incubated tubes (16.7 mM ± 0.6) for all 45 positive MPN cultures analyzed in duplicate (mean ± SEM, $P < 0.001$). Repressurization was done during the enumeration protocol, which precluded the evaluation of the reaction stoichiometry. Cell morphologies observed in enrichment broths were similar to that of *A. ruminis* 190A4 in addition to a long rod and a coccus.

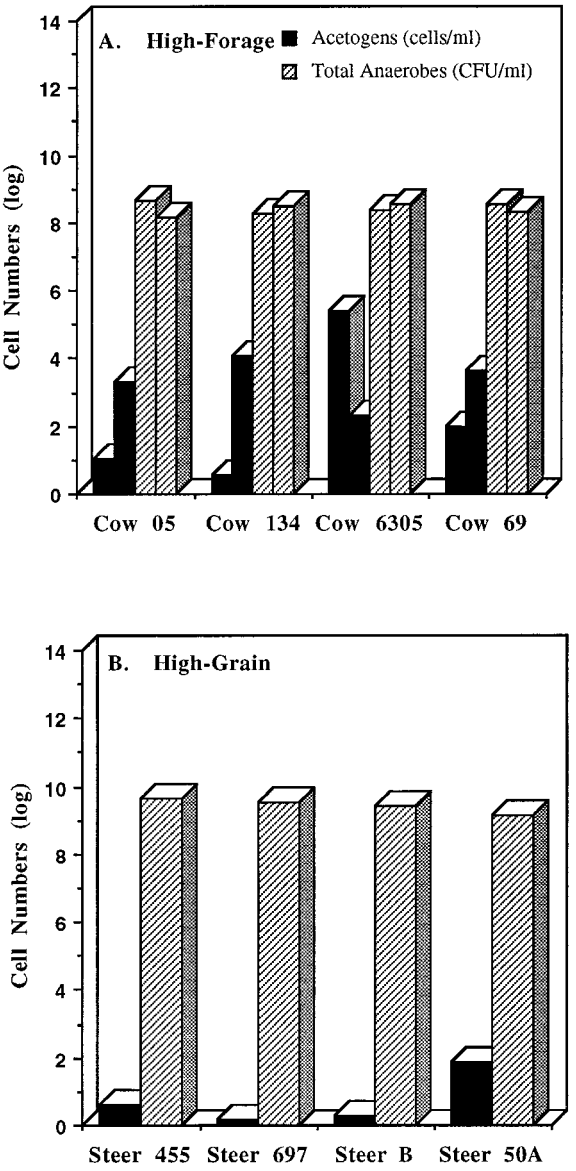

FIG. 1. Enumeration of acetogenic and total viable anaerobic bacteria from the bovine rumen. Acetogenic bacteria were enriched on a rumen fluid-based medium containing 2.5 mM BES and enumerated by the MPN method. (A) Enumeration from ruminal contents of cattle fed a high-forage diet. Bars of the same pattern represent duplicate enumeration from the same animal done within 1 to 5 months. (B) Enumeration from ruminal contents of cattle fed a high-grain diet. Each bar represents one enumeration from one animal.

TABLE 1. Endogenous ruminal acetogenic activity

Concn (mM) of BES added to ruminal contents[a]	Gas phase	Mean concn (mmol) ± SEM (n = 3)	
		[1- or 2-^{13}C]acetate-carbon	[U-^{13}C]acetate-carbon
5	H_2	0.26 ± 0.018	0.24 ± 0.002
	N_2	0.08 ± 0.004	0.04 ± 0.001
0	H_2	0.06 ± 0.003	0.04 ± 0.003
	N_2	0.06 ± 0.000	0.02 ± 0.001

[a] Reaction mixtures contained 9 ml of ruminal contents, 5 mM BES, 0.8 mmol of $NaH^{13}CO_3$, 100% H_2 (6.0 mmol) or 100% N_2 gas phase, and other reagents as indicated in Materials and Methods. Ruminal contents were incubated for 45 h. At time zero, the [1- or 2-^{13}C]acetate-carbon concentration was 0.04 ± 0.001 mmol and the [U-^{13}C]acetate-carbon concentration was 0.02 ± 0.001 mmol.

Endogenous rumen acetogenic activity. Activity of endogenous rumen acetogenic bacteria was investigated by incubating ruminal contents with 100% H_2 gas phase and $NaH^{13}CO_3$. When methanogenic bacteria were inhibited by BES, 0.18 mmol of net [1- or 2-^{13}C]acetate-carbon and 0.2 mmol of net [U-^{13}C]acetate-carbon accumulated, indicating ruminal acetogenic activity (Table 1). H_2 and CO_2 were in excess during the entire incubation period, and methane was not detected in the headspace gas (data not shown). The concentration of residual gas was not determined. There was a specific enhancement of acetogenesis, as shown by ^{13}C incorporation into acetate under H_2 compared to N_2, presumably due to limiting endogenous reducing equivalents (i.e., H_2 or formate). Only trace amounts of ^{13}C were associated with propionate and butyrate (<0.02 mmol).

Activity of endogenous ruminal acetogenic bacteria was dissipated in H_2-incubated vials which did not receive BES. Zero net [1- or 2-^{13}C]acetate and 0.02 mmol of net [U-^{13}C]acetate accumulated (Table 1). CO_2 was completely utilized during the incubation of ruminal contents without BES; however, the fate of $NaH^{13}CO_3$ was not determined. These vials contained residual H_2 and methane in the gas phase after the 45 h of incubation. Quantitation of H_2 and methane was not assessed in these vials. There was a specific enhancement of methane accumulation under H_2 compared to N_2 gas phase, implying a limitation in available reducing equivalents (data not shown).

Role of population density in reductive ruminal acetogenic activity: in vitro incubations with nonlimiting concentration of H_2. Whether endogenous ruminal acetogenesis could be limited by the population density of acetogenic bacteria was addressed in the following experiments. Ruminal contents were incubated with 100% H_2 gas phase, $NaH^{13}CO_3$, BES, and an *A. ruminis* 190A4 inoculum. When methanogenic bacteria were inhibited by BES, accumulation of acetate-carbon was positively correlated with the population density of added *A. ruminis* 190A4 from 10^3 to 10^6 CFU/ml (Fig. 2). Maximum levels of $^{13}CO_2$ fixed into acetate, as indicated by the final concentration of carbon in singly and doubly labeled acetate, were observed when the final concentration of *A. ruminis* 190A4 added to ruminal contents was 10^5 to 10^7 CFU/ml of ruminal contents. An average of 0.39 mmol of total acetate (equivalent to 0.78 mmol of acetate-carbon) accumulated from the utilization of approximately 1.7 mmol of H_2. Based on accumulation of doubly and singly labeled acetate, levels of acetogenic activity for these levels of inoculation were approximately 50% greater than that found for H_2-supported, net endogenous acetogenic activity in the presence of BES (Table 1). The concentration of *A. ruminis* 190A4 required for enhancement of rumen acetogenic activity was equal to or greater than the acetogen population enumerated in animals fed the high-forage diet (Fig. 1A). Growth of *A. ruminis* 190A4 was not negatively affected by a BES concentration of ≤5 mM (data not shown). Methane was not detected in these vials which contained BES (data not shown). Low levels of propionate and butyrate accumulated; however, only trace amounts (<0.02 mmol) of ^{13}C were associated with propionate and butyrate. All values in Fig. 2 represent strictly H_2-dependent fixation products since the amount of CO_2 incorporated into a particular product under H_2 was corrected for the accumulation of the same product under an N_2 atmosphere.

When BES was not present in the incubation vials containing excess H_2 as described above, acetogenesis was stimulated only at the highest concentration of *A. ruminis* 190A4 tested (Fig. 3). An increase in [1- or 2-^{13}C]- and [U-^{13}C]acetate-carbon at the expense of methane accumulation was observed when the concentration of *A. ruminis* 190A4 added to rumen-like incubations was 2.2×10^7 CFU/ml of ruminal contents ($P < 0.05$). This acetogen concentration was 100-fold greater than that found by enumeration studies (Fig. 1A). Reduction in methane concentration presumably resulted from diminished availability of CO_2 due to its fixation into acetate. CO_2 was completely utilized in all incubations, while H_2 was present in excess in all vials (data not shown). Approximately 0.8 mmol of $NaH^{13}CO_3$ was utilized for the production of 0.6 mmol of methane and 0.3 mmol of acetate-carbon. Low levels of formate, propionate, and butyrate accumulated during the incubation; however, only trace amounts of ^{13}C were associated with propionate and butyrate (<0.02 mmol). Initial methanogenic (1.7×10^8 cells/ml) and total viable anaerobic (8.4×10^8 CFU/ml) population densities were at least 10-fold higher than the highest concentration of *A. ruminis* 190A4 added to rumen-like incubations.

Role of H_2 concentration in ruminal reductive acetogenic activity: in vitro incubations with limiting concentration of H_2. To assess the competitiveness of acetogenic bacteria in rumen-

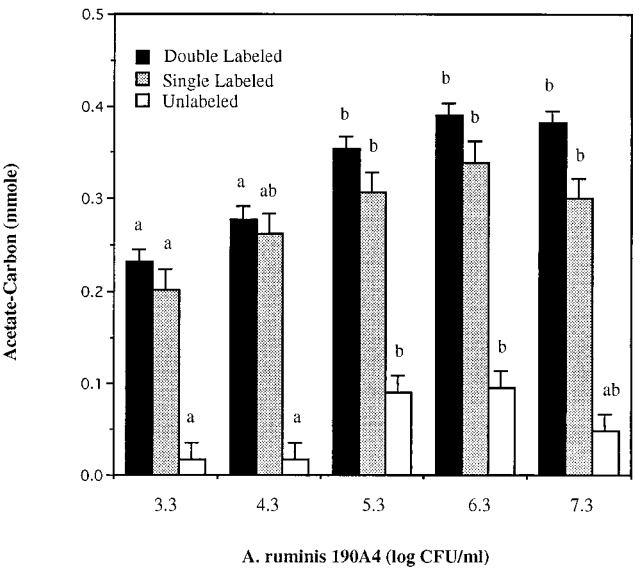

FIG. 2. H_2-dependent [^{13}C]acetate accumulation in rumen-like incubations amended with *A. ruminis* 190A4 and BES. Reaction mixtures contained 9 ml of ruminal contents, 5 mM BES, 0.8 mmol of $NaH^{13}CO_3$, *A. ruminis* 190A4 inoculum, 100% H_2 gas phase (6.0 mmol), and other reagents as indicated in Materials and Methods. Vials were incubated for 48 h at 200 rpm and 39°C prior to sample collection for [^{13}C]acetate analysis by MS. Means of the same bar pattern with different letters are different ($P < 0.025$). Data are presented as means + SEMs for three determinations.

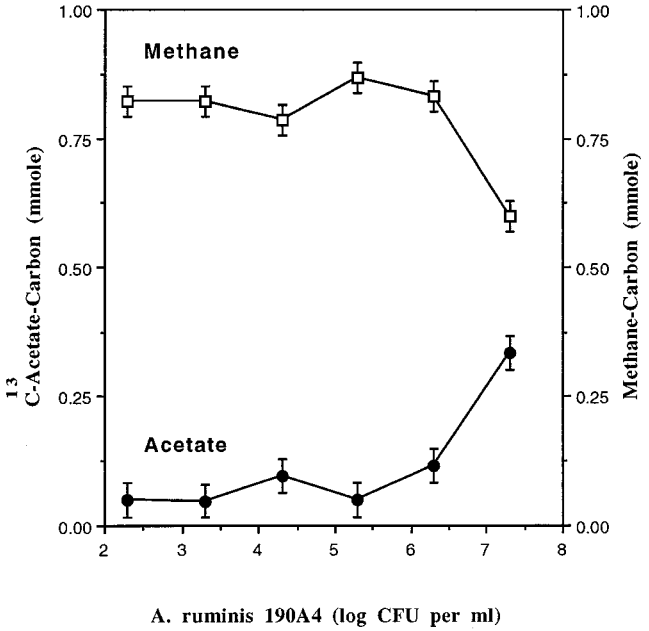

FIG. 3. H_2-dependent [^{13}C]acetate and methane accumulation in rumen-like incubations with *A. ruminis* 190A4 and active methanogenic bacteria (without BES). Reaction mixtures contained 9 ml of ruminal contents, 0.8 mmol of $NaH^{13}CO_3$, *A. ruminis* 190A4 inoculum, 100% H_2 gas phase (6.0 mmol), and other reagents as indicated in Materials and Methods. Vials were incubated for 48 h at 200 rpm and 39°C prior to sample collection for methane and [^{13}C]acetate analysis by GC and MS, respectively. [^{13}C]acetate-carbon is the summation of [1- or 2-^{13}C]- and [U-^{13}C]acetate-carbon. Each point represents the mean ± SEM for three determinations.

like conditions, in vitro incubations were conducted with an ecologically common source of H_2. The accumulation of H_2 in the headspace gas over time was measured in vials containing ruminal contents incubated with 100% N_2 gas phase, $NaH^{13}CO_3$, and alfalfa in the presence or absence of BES and/or *A. ruminis* 190A4. Alfalfa was added as a slowly degradable carbohydrate source for the production of a rumen-like H_2 concentration. Under conditions with active methanogenesis (without BES), H_2 was generated from the alfalfa fermentation and utilized for methanogenesis (Fig. 4). These vials which supported methanogenic activity contained approximately 0.002 mmol of gaseous H_2, which was equivalent to an H_2 equilibrium concentration in the headspace gas of less than 400 ppm during the entire 43-h incubation. This H_2 concentration was independent of added *A. ruminis* 190A4 (Fig. 4A; $P > 0.95$). The H_2 equilibrium concentration was considered to be the balance between H_2 production and utilization. Methanogenic activity was evidenced by the accumulation of headspace methane and also found to be similar for vials with or without 4.1×10^8 *A. ruminis* 190A4 CFU/ml (Fig. 4B; $P > 0.9$), suggesting minimal H_2/CO_2-supported activity of *A. ruminis* 190A4 in methanogenesis-supportive incubations. Furthermore, singly or doubly labeled acetate-carbon did not accumulate in vials with active methanogenesis and added *A. ruminis* 190A4 to levels above those measured in control vials without the addition of *A. ruminis* 190A4 (Table 2; $P > 0.2$).

Vials containing ruminal contents in which methane production was inhibited (with BES) had significantly higher H_2 equilibrium concentrations in the headspace gas than methanogenesis-supportive vials (Fig. 4A; $P < 0.001$). Singly and doubly labeled acetate accumulated in these vials to levels above those in vials without the addition of *A. ruminis* 190A4, indicating CO_2 fixation activity of *A. ruminis* 190A4 in ruminal contents amended with BES (Table 2). In vials containing BES and 10^8 *A. ruminis* 190A4 CFU/ml, the H_2 equilibrium concentration in the headspace gas was approximately 4,800 ppm throughout the 43-h incubation (Fig. 4A). Accumulation of H_2 was inversely proportional to the concentration of *A. ruminis* 190A4 up to 10^6 CFU/ml, indicating that the H_2-consuming capacity of ruminal contents was dependent on the population density of *A. ruminis* 190A4 (data not shown). With *A. ruminis* 190A4 densities greater than or equal to 10^6 CFU/ml in vials amended with BES, the headspace contained 0.023 mmol of H_2 (equivalent to approximately 4,800 ppm of H_2), and gas production was independent of *A. ruminis* 190A4 concentration ($P > 0.2$). This result suggests a saturation of *A. ruminis* 190A4 capacity for H_2 utilization.

In contrast, H_2 accumulated over time in vials which re-

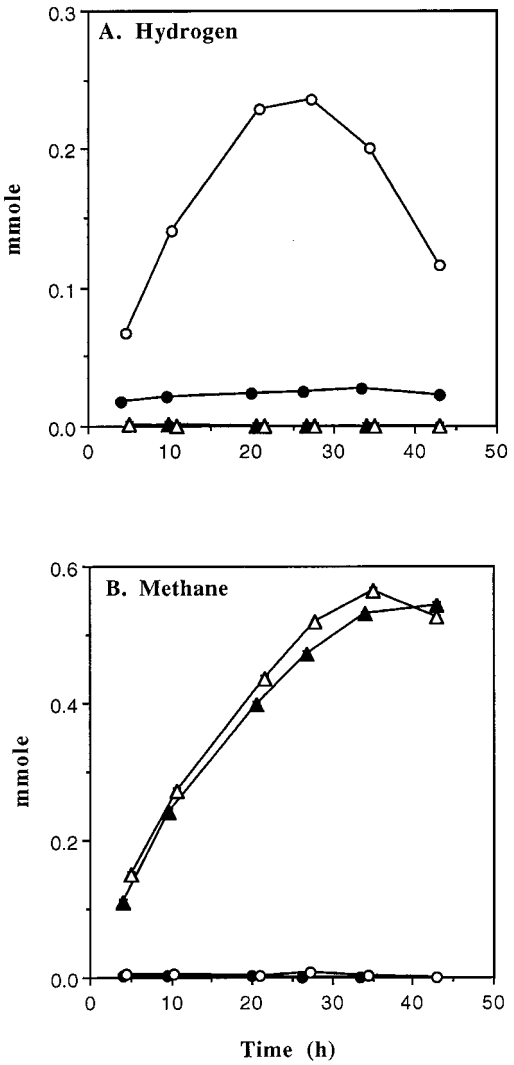

FIG. 4. Hydrogen and methane concentrations over time in vials with ruminal contents incubated with alfalfa and 100% N_2. Reaction mixtures contained 9 ml of ruminal contents, with or without 5 mM BES, 0.82 mmol of $NaH^{13}CO_3$, *A. ruminis* 190A4 inoculum, 0.38 g of alfalfa, and other reagents as indicated in Materials and Methods. When added, the final concentration of *A. ruminis* was 4.1×10^8 CFU/ml. Symbols: open circles, with BES; closed circles, with BES and added *A. ruminis* 190A4; open triangles, without BES and without addition of *A. ruminis* 190A4; closed triangles, without BES and with added *A. ruminis* 190A4. Each point represents the mean ± SEM for three determinations. Standard errors were plotted but are too small to be visible on the graph.

TABLE 2. [^{13}C]acetate accumulation in ruminal contents incubated with alfalfa and 100% N_2[a]

Addition to ruminal contents		Mean concn (mmol) ± SEM (n = 3)	
BES	*A. ruminis* 190A4 (CFU/ml)	[1- or 2-^{13}C]acetate-carbon	[U-^{13}C]acetate-carbon
+	4.1×10^8	0.35 ± 0.008^b	0.08 ± 0.000^b
	None	0.17 ± 0.008^c	0.04 ± 0.002^c
−	4.1×10^8	0.13 ± 0.002	0.04 ± 0.002
	None	0.14 ± 0.004	0.03 ± 0.002

[a] Reaction mixtures were as described in the legend to Fig. 4.
[b,c] Means for BES treatment and ^{13}C-labeling pattern with different superscripts are different ($P < 0.001$).

ceived BES but no added *A. ruminis* 190A4, suggesting minimal nonmethanogenic H_2-consuming activity in ruminal contents (Fig. 4A). In these vials, H_2 consumption finally exceeded production after 27 h of incubation, indicating increased H_2 consumption by, presumably, endogenous H_2/CO_2-supported acetogenic bacteria or decreased production of H_2 from the alfalfa fermentation. Increased H_2 consumption may be related to an increase in population density of endogenous H_2/CO_2-consuming acetogenic bacteria during the fermentation. H_2/CO_2-consuming acetogenic bacteria were not enumerated in ruminal contents at the time of this experiment but were previously found to be present at a density of 3.5×10^1 to 2.6×10^5 cells/ml for the same animals fed a high-forage diet (Fig. 1A). All values in Fig. 4 represent accumulation of fermentation products under a 100% N_2 gas phase and are not confirmed to be H_2-dependent fixation products since it was not possible to have a control incubation in which the alfalfa fermentation did not produce H_2.

Pure-culture H_2 threshold studies. Competition for H_2 can be partially explained by the threshold model, which states that the successful organism keeps the H_2 partial pressure below the level necessary to allow H_2 oxidation by competitors. For this reason, hydrogen threshold values for selected acetogens and a methanogen were determined (Table 3). The H_2 threshold concentrations for *A. ruminis* 190A4 and the methanogen were consistent with the equilibrium concentrations found in in vitro incubations with an alfalfa fermentation providing H_2 in the presence and absence of BES, respectively (Fig. 4A). When *S. termitida* was incubated under N_2-CO_2, H_2 was produced to a level of 1,200 ppm. It appears that *S. termitida* produces H_2 to a level near its threshold. As with *S. termitida*, *A. woodii* can produce H_2 (328 ppm) up to its threshold level. Approximately 40 ppm of H_2 was found in all inoculated tubes analyzed just after initial pressurization with N_2-CO_2. This value is at most only 35% of the lowest threshold value presented. In incubated, uninoculated tubes pressurized with N_2-CO_2, there was no abiological production of H_2 (data not shown).

DISCUSSION

In this study, we have demonstrated that reductive acetogenic bacteria are inhabitants of the rumen ecosystem yet have negligible endogenous H_2/CO_2-consuming activity. Reductive acetogenic activity was enhanced in rumen-like incubations when (i) an axenic culture of the rumen acetogen *A. ruminis* 190A4 was added under conditions of H_2 excess or (ii) methanogenesis was selectively inhibited by BES and *A. ruminis* 190A4 was added to incubations with a limiting concentration of H_2. These data, in addition to those from pure-culture H_2 threshold studies, confirm that ruminal methanogenic bacteria limit reductive acetogenesis by lowering the H_2 partial pressure below the minimum level necessary for H_2 consumption by a ruminal acetogen, *A. ruminis* 190A4.

H_2/CO_2-supported acetogenic bacteria in the rumen were enumerated (Fig. 1A and B) and found to be present at concentrations greater than those found in the feed and water (31a). Bryant suggested that the number of a given species present in the rumen compared to its numbers in the feed and water consumed is probably the best measure of whether an organism is a true rumen microorganism (8). A large variability was observed within duplicate enrichments from animals 05, 134, 6305, and 69, which was believed to be due to sample variation instead of the MPN technique, since good repeatability was associated with the latter (31a). Leedle and Greening found H_2/CO_2-utilizing acidogenic bacteria in the rumens of steers fed a high-forage (3.9×10^8 cells per g of ruminal contents) or a high-grain (8.7×10^8 cells per g of ruminal contents) diet (30). In contrast, we found H_2/CO_2-supported ruminal acetogenic bacteria at a concentration at least 1,000-fold lower than that found by Leedle and Greening (30). This discrepancy could be explained by sample variation due to a rumen sample obtained via stomach tube versus through a cannula (30) or an overestimation of H_2/CO_2-consuming acidogens due to enumeration via bromocresol green-staining colonies. We found that beef cows fed a hay diet supported a greater population density of ruminal reductive acetogenic bacteria than did steers fed a finishing diet. Some of the determinants affecting the population density of H_2/CO_2-consuming ruminal acetogenic bacteria may be the presence of alternate energy sources in the hay diet, inhibition by monensin in the high-grain diet, and/or lower rumen pH with the high-grain diet. The rumen pH of animals fed the high-grain diet (<6.0) was lower than that found for animals fed the high-forage diet (<6.5) because starch is a readily fermentable substrate (3). H_2/CO_2-consuming acetogenic bacteria have pH optima close to 7.0. Optimal pHs for *A. ruminis* and *Eubacterium limosum* are pH 6.8 and 7.2, respectively; therefore, a lower rumen pH would likely be inhibitory to the growth of acetogenic bacteria (22, 30). The lack of H_2/CO_2-supported acetogenic bacteria in enrichments from high-grain diet rumen samples done in pH 6.1 medium support this hypothesis. Also, monensin disrupts

TABLE 3. Thresholds for hydrogen of selected H_2-consuming bacteria

Microorganism	Incubation (temp [°C])	Gas composition	No. of determinations	Mean H_2 (ppm)[a] ± SEM
A. ruminis 139B	+ (38)	H_2-N_2-CO_2[b]	33	4,660 ± 39
	+ (38)	N_2-CO_2[c]	18	147 ± 15
	−	N_2-CO_2	4[d]	39 ± 3
A. ruminis 190A4	+ (38)	H_2-N_2-CO_2	34	3,830 ± 69
	+ (38)	N_2-CO_2	13	68 ± 22
	−	N_2-CO_2	4[d]	41 ± 1
Methanogen 10-16B	+ (38)	H_2-N_2-CO_2	32	126 ± 4
	+ (38)	N_2-CO_2	ND[e]	ND
S. termitida	+ (30)	H_2-N_2-CO_2	20	871 ± 88
	+ (30)	N_2-CO_2	15	1,200 ± 95
	−	N_2-CO_2	4[d]	12 ± 1
A. woodii	+ (30)	H_2-N_2-CO_2	33	362 ± 9
	+ (30)	N_2-CO_2	17	328 ± 14
	−	N_2-CO_2	4[d]	35 ± 3

[a] Gas-phase concentration at the end of incubation. To convert to micromolar H_2 in the liquid phase, multiply parts per million of H_2 by 7.62×10^{-4} and 7.36×10^{-4} for tubes incubated at 30 and 38°C, respectively (45).
[b] 1.2:75.2:23.6 (vol%).
[c] 80:20 (vol%).
[d] Analyzed for H_2 immediately after initial pressurization with N_2-CO_2.
[e] ND, not determined.

K^+ and Na^+ gradients, which are usually associated with inhibition of ruminal gram-positive bacteria (39). *A. ruminis* tends to be gram variable but stains gram positive in 24-h cultures (25).

In in vitro incubations with a nonlimiting concentration of H_2, endogenous acetogenic activity was not detected in rumen-like incubations unless methanogenesis was selectively inhibited by BES (Table 1). This activity in the presence of BES could be further enhanced by the addition of an axenic culture of the rumen acetogen *A. ruminis* 190A4 (Fig. 2). Reductive acetogenic activity in rumen-like incubations without BES was dependent on the concentration of added *A. ruminis* 190A4 under conditions of H_2 excess (Fig. 3). Simultaneous activity of acetogenic and methanogenic bacteria was indicated only when the population density of *A. ruminis* 190A4 was 10^7 CFU/ml, nearly approaching the rumen concentration of methanogenic bacteria (10^8 cells/ml). Hence, the population density of *A. ruminis* 190A4 dramatically influenced the accumulation of acetate resulting from H_2/CO_2-utilizing activity. These results are similar to those found by Nollet et al. upon addition of the acetogen *Peptostreptococcus productus* to ruminal contents (36).

Singly labeled acetate most likely represents a total synthesis of acetate from CO_2 arising from the fixation of 1 mol of $^{12}CO_2$ and 1 mol of $^{13}CO_2$ into [1- or 2-^{13}C]acetate (41). Attempts were made to remove soluble $^{12}CO_2$ from ruminal contents; however, complete removal could not be attained ($<$0.01 mmol of residual CO_2). In addition, there was presumably production of $^{12}CO_2$ from residual substrates in ruminal contents. The [1- or 2-^{13}C]acetate could also arise from an exchange reaction between $^{12}CO_2$ and the carboxyl or methyl group of [U-^{13}C] acetate or [U-^{12}C]acetate (46). We found that $<$15% $^{12}CO_2$ is exchanged with [U-^{13}]acetate in ruminal contents, and thus only a small portion of [^{13}C]acetate may not represent a total synthesis of acetate. For this study, doubly labeled acetate was used to confirm total synthesis of acetate from CO_2 and the summation of singly and doubly labeled acetate was used to quantitate H_2/CO_2-utilizing acetogenic activity. Our ability to detect CO_2 fixation activity by MS was verified by analysis of culture supernatants of *A. ruminis* 190A4 incubated in pure culture with H_2 and $NaH^{13}CO_3$. Mass spectra revealed explicitly the accumulation of [U-^{13}C]acetate, which was further confirmed by nuclear magnetic resonance analysis (31a).

It has been hypothesized, on the basis of nonruminal acetogenic isolates, that ruminal acetogenic bacteria cannot compete with ruminal methanogenic bacteria because they have less effective H_2-scavenging ability. We have shown that two representative ruminal acetogens, *A. ruminis* 139B and *A. ruminis* 190A4, have H_2 threshold concentrations which are 30- to 37-fold greater than that of the methanogen 10-16B (Table 3) and 3-fold higher than the median ruminal in situ H_2 concentration (1.0 μM in the aqueous phase, which is equivalent to 1,360 ppm in the atmospheric phase) (18, 27, 38, 42, 45). The ruminal acetogens had 4- to 13-fold-higher threshold values compared to nonruminal H_2-utilizing acetogenic bacteria. This may be related to the fact that ruminal acetogens have not been selected in vivo on the basis of H_2-scavenging ability. For example, *S. termitida* had a lower H_2 threshold value than *A. ruminis* and was isolated from a wood-eating termite hindgut, where H_2-dependent acetogenesis is the dominant H_2 sink reaction (7). H_2 threshold values for *S. termitida* and *A. woodii* were in close agreement with the values of 830 and 520 ppm, respectively, reported by Cord-Ruwisch et al. (14). The pure-culture H_2 threshold data indicate that the two rumen acetogens have poorer H_2-scavenging ability than methanogens in pure culture. Supporting this conclusion, in in vitro incubations with a limiting concentration of H_2, methanogenic bacteria and *A. ruminis* 190A4 maintained H_2 concentrations of approximately 400 and 4,800 ppm, respectively (Fig. 4), which are similar to their H_2 thresholds. This finding demonstrated that ruminal methanogenic bacteria limited acetogenesis by lowering the H_2 partial pressure to a level insufficient for H_2 utilization by *A. ruminis* 190A4. This observation agrees well with our understanding of methanogenesis as the predominant H_2 sink in the rumen (26, 27). Redirection of ruminal H_2 disposal seems to require a strategy for compromising H_2 consumption by ruminal methanogens and selection of a ruminal acetogen with a H_2-scavenging ability approaching that of ruminal methanogens. Failure of an alternative H_2 disposal strategy to achieve equally low ruminal in situ H_2 concentrations could be deleterious to the thermodynamics of interspecies H_2 transfer and, hence, degradative activity of ruminal fermentative bacteria.

In summary, the coexistence of methanogenic and reductive acetogenic bacteria in the rumen suggests that the acetogens grow on substrates other than H_2 and CO_2 (i.e., noncompetitive substrates such as organic compounds) in situ or there is an abundance of competitive substrates (i.e., H_2) in the rumen (33, 37) due to diurnal fluctuations of H_2 and/or juxtapositioning between H_2-producing and H_2-consuming microorganisms (3, 18, 38, 42).

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