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Effect of 2-Bromoethanesulfonic Acid and *Peptostreptococcus productus* ATCC 35244 Addition on Stimulation of Reductive Acetogenesis in the Ruminal Ecosystem by Selective Inhibition of Methanogenesis

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Evidence is provided that reductive acetogenesis can be stimulated in ruminal samples during short-term (24-h) incubations when methanogenesis is inhibited selectively. While addition of the reductive acetogen *Peptostreptococcus productus* ATCC 35244 alone had no significant influence on CH_4 and volatile fatty acid (VFA) production in ruminal samples, the addition of this strain together with 2-bromoethanesulfonic acid (BES) (final concentration, 0.01 or 0.03 mM) resulted in stimulation of acetic acid production and H_2 consumption. Since acetate production exceeded amounts that could be attributed to reductive acetogenesis, as measured by H_2 consumption, it was found that *P. productus* also fermented C_6 units (glucose and fructose) heterotrophically to mainly acetate (>99% of the total VFA). Using $^{14}CH_3COOH$, we concluded that addition of BES and BES plus *P. productus* did not alter the consumption of acetate in ruminal samples. The addition of *P. productus* to BES-treated ruminal samples caused supplemental inhibition of CH_4 production and stimulation of VFA production, representing a possible energy gain of about 13 to 15%.

During the last few decades, it has been a challenge for scientists active in the field of animal production to improve the rumen fermentation process. Depending on the type of diet, up to 5 to 15% of the ingested gross energy can be lost through methanogenesis in the rumen (10, 34, 39). Attempts have been made in the past to reduce this energy loss by inhibiting CH_4 production, either through the addition of antibiotics, ionophores, or halogenated methane analogs or by changing the feed composition. The additives decrease methanogenesis in the rumen, but unfortunately they introduce the risk of drug residues in edible tissues and in milk. Recently, it has been hypothesized that rumen fermentation could be improved by stimulating reductive acetogenesis and inhibiting CH_4 production (11, 23). Reductive acetogenic strains have been isolated from different anaerobic habitats, including lakes, mud, nonmarine anoxic environments, sewage sludge, and even soils (2, 16, 25, 28, 36). The presence of reductive acetogens in rats and in human gastrointestinal ecosystems (15, 26, 29, 33) has led to the discovery of the presence of reductive acetogenic strains in the rumen (18, 21, 27).

Reductive acetogens reduce 2 mol of CO_2 to acetate by oxidation of H_2, as follows (16, 33, 41):

$$2\,CO_2 + 4\,H_2 \rightarrow CH_3COOH + 2\,H_2O$$

Since reductive acetogens consume H_2, in the rumen they are in direct competition with the hydrogenotrophic methanogens, which utilize H_2 to reduce CO_2 to CH_4, as follows (32, 40):

$$CO_2 + 4\,H_2 \rightarrow 2\,CH_4 + 2\,H_2O$$

However, since the affinity of the methanogens for H_2 is 10 to 100 times higher than the affinity of the reductive acetogens, the H_2 partial pressure in the rumen is normally too low to allow the reductive acetogens to grow autotrophically (i.e., on H_2-CO_2) (8, 21). Since reductive acetogens are able to grow mixotrophically (i.e., they can grow heterotrophically in the absence of H_2-CO_2 [7]), they can persist in almost every anaerobic ecosystem. Their heterotrophic growth is characterized by the fermentation of monosaccharides to mainly acetate, as follows (13):

$$C_6H_{12}O_6 + H_2O \rightarrow CH_3COCOOH + CH_3COOH + CO_2 + 3\,H_2O$$

$$CH_3COCOOH + CO_2 + 3\,H_2 \rightarrow 2\,CH_3COOH + H_2O$$

$$C_6H_{12}O_6 \rightarrow 3\,CH_3COOH$$

However, when no methanogenic activity is present, their autotrophic characteristics are activated.

If CH_4 production could be partially or completely replaced by reductive acetogenesis in the rumen, there would be the following three benefits: (i) there would be a reduction in energy loss via CH_4 production, (ii) energy-rich compounds (acetate) would be produced from CO_2, and (iii) a greenhouse gas would be eliminated. Activation of reductive acetogenesis in the rumen by selectively inhibiting CH_4 production and introduction of allochthonous reductive acetogenic species have been suggested previously (14, 23, 42), but have not been examined systematically (40). Therefore, in this study we investigated the possibility of stimulating reductive acetogenesis by adding a reductive acetogenic strain, *Peptostreptococcus productus* ATCC 35244, in absence or presence of a selective inhibitor of CH_4 production. Also, the influence of the addition of this reductive acetogenic strain on ruminal fermentation processes, particularly volatile fatty acid (VFA) production, was studied.

MATERIALS AND METHODS

Source of the strain. *P. productus* ATCC 35244 was obtained from the American Type Culture Collection. This strain was isolated from sewage digester

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sludge, was originally designated U-1 (29), and was further characterized by Geerligs et al. (17). *P. productus* was used in the experiments since its ecological parameters (e.g., optimum pH and temperature for growth) were identical to conditions in the rumen (17).

Heterotrophic cultivation of *P. productus* ATCC 35244. *P. productus* ATCC 35244 was cultivated in an anaerobic medium called RACh medium. This medium was prepared by boiling distilled water for 5 min and then cooling it on ice under oxygen-free nitrogen gas as described previously (3, 22). The following components were added after cooling: 30 g of Bacto Peptone (Difco) per liter, 5 g of yeast extract per liter, 2.5 g of K_2HPO_4 per liter, and 2.0 g of KH_2PO_4 per liter. The medium was reduced by adding 0.5 g of cysteine-HCl per liter. One milliliter of a resurazin solution (0.1%, wt/vol) was added as a redox indicator. The medium was dispensed under a continuous oxygen-free nitrogen gas flow into 27-ml cultivation tubes. Enough cooked meat medium (Oxoid) was put in these tubes previously to yield a final concentration of 10% (wt/vol). The tubes were sealed under a 100% N_2 atmosphere with butyl rubber stoppers, crimped with aluminum seals, and autoclaved.

P. productus was cultivated in the tubes by inoculating them with subcultures of a grown culture (5% [final concentration] in solution) by using sterile syringes and needles which had previously been flushed with sterile nitrogen to eliminate any oxygen present in the needles. Cultures were grown by incubating them at 37°C for 48 h. The final densities were about $\log_{10}$ 8 to 9 CFU/ml.

Autotrophic cultivation of *P. productus* ATCC 35244. The medium used for cultivation of *P. productus* ATCC 35244 under autotrophic conditions (RACa medium) was based on a previously described medium (43). First, Milli-Q water was boiled for 5 min and then cooled on ice under oxygen-free nitrogen gas. After cooling, the following components were added: 7.5 g of $NaHCO_3$ per liter, 1.0 g of NH_4Cl per liter, 0.3 g of K_2HPO_4, per liter, 0.3 g of KH_2PO_4 per liter, 0.61 g of NaCl per liter, 0.3 g of $(NH_4)_2SO_4$ per liter, and 0.5 g of HCOONa per liter. The medium was reduced by adding 0.5 g of cysteine-HCl per liter. One milliliter of resurazin (0.1% wt/vol) was added as a redox indicator. The medium was dispensed into 27-ml cultivation tubes under a constant flow of an H_2-CO_2 (80:20, vol/vol) gas mixture. The tubes were closed with butyl rubber stoppers and crimped with aluminum seals. The pressure in the headspaces of the tubes was increased to 202 kPa by using the H_2-CO_2 gas mixture, after which the tubes were autoclaved.

P. productus was cultivated autotrophically in RACa medium by inoculating the medium with a subculture of the strain grown in RACh medium (inoculum size, 1% [final concentration]) by using sterile syringes and needles previously flushed with sterile nitrogen gas. The culture tubes were incubated at 37°C for 72 h, after which subcultures were transferred to new culture tubes containing RACa medium and H_2-CO_2 as the headspace gas at a pressure of 202 kPa. This procedure was repeated five times to eliminate all carbon sources except HCO_3^-. Autotrophic growth was determined by monitoring turbidity and the decrease in pressure inside the inoculated cultivation tubes and by measuring the H_2 and CO_2 concentrations in the headspaces of the tubes.

Preparation of anaerobic BES solutions. Aqueous 2-bromoethanesulfonic acid (BES) solutions were prepared by adding the sodium salt of BES to boiled and cooled Milli-Q water and then adding 1 ml of resurazin (0.1%, wt/vol) per liter and 0.5 g of cysteine-HCl per liter. The BES solutions were filter sterilized and transferred into closed sterile 40-ml penicillin flasks containing oxygen-free N_2 (10^5 Pa).

Effect of BES on heterotrophic growth. *P. productus* ATCC 35244 was grown for 48 h at 37°C in culture tubes containing 7.9 ml of RACh medium prepared as described above. After 48 h, 0.1-ml portions of different anaerobic filter-sterilized BES solutions were added to the culture tubes by using sterile syringes to obtain final BES concentrations of 0.00, 0.01, and 0.03 mM. The tubes were vigorously shaken for 10 s and then incubated without shaking at 37°C. VFA concentrations were determined 0, 24, 48, and 72 h after BES was added by removing 0.25-ml samples with sterile syringes and needles, while bacterial counts were determined by preparing 10-fold dilutions in sterile anaerobic phosphate buffer (0.1 M P-phosphate, reduced by adding 0.1% [wt/vol] sodium thioglycolate) and then plating the preparations onto petri dishes containing brain heart infusion agar (Oxoid). Dilution and plating were performed in an anaerobic chamber containing 8% H_2, 8% CO_2, and 84% N_2. After plating, the petri dishes were transferred into an anaerobic jar, made anaerobic with an Anoxomat system (MART), and incubated at 37°C for 48 h before counting. Experiments were conducted in triplicate and repeated to confirm results.

Effect of BES on autotrophic growth. One-hundred-microliter autotrophically growing *P. productus* ATCC 35244 subcultures were transferred into culture tubes containing 4.8 ml of sterile RACa medium under 80% H_2–20% CO_2 (total pressure, 202 kPa) and incubated for 72 h at 37°C. To avoid depletion of H_2 and CO_2, the pressure in the headspaces of the culture tubes was restored to 202 kPa every 24 h by using an 80% H_2–20% CO_2 gas mixture made sterile by passing it through a 0.22-μm-pore-size filter. One-hundred-microliter portions of different anaerobic filter-sterilized BES solutions were added to the culture tubes by using sterile syringes and needles; the final concentrations of BES were 0.00, 0.01, and 0.03 mM. The tubes were shaken vigorously for 10 s and then incubated without shaking at 37°C. At time zero and 24 h, samples were removed with sterile syringes and needles to determine the amounts of VFA. Gas samples were taken at the end of incubation to determine the H_2 concentration. Experiments were conducted in triplicate and repeated to confirm results.

Collection of ruminal samples. Ruminal samples were obtained from a 3-year-old rumen-fistulated ram. At 0900 and 1600 h the ram was fed 300 g of hay and 300 g of concentrate (31). The samples were collected at 0930 h, sieved through a metal sieve (mesh size, 1 mm) under a constant CO_2 flow, and transported to the lab under CO_2 at 37°C in a thermoflask. The time interval between collection of samples and use in experiments never exceeded 1 h.

Addition of BES and/or *P. productus* ATCC 35244 to ruminal samples. Eighteen 125-ml penicillin flasks were filled under a CO_2 flow with 33 ml of CO_2-saturated, sterilized Burroughs solution (9) and 10 ml of sieved ruminal sample. The pH of the Burroughs solution was previously adjusted to 7.2 with 2 M NaOH. Five milligrams of N-NH_4^+ was added to every flask by adding 1 ml of an NH_4HCO_3 solution, and 500 mg of hay (diameter, <2 mm [31]) was added as a carbon source. The flasks were flushed for 10 min with 100% CO_2, closed with butyl rubber stoppers, and crimped with aluminum seals.

The 18 bottles were divided into three groups of 6 bottles. Each group received a different dose of BES (final concentrations, 0.00, 0.01, and 0.03 mM), which was added by injecting 1 ml of a sterile, anaerobic BES solution through the septum of each bottle. Three of the six bottles in each group were inoculated with 5 ml of a culture of *P. productus* ATCC 35244 grown in RACh medium, which resulted in final concentrations of $\log_{10}$ 7.50 ± 0.15 CFU/ml, while the other three bottles received 5 ml of sterilized RACh medium. The bottles were vigorously shaken for 10 s to mix all of the components and were incubated in a shaking warm water bath (165 rpm) at 37°C. Fermentation was stopped after 24 h by injecting 1 ml of 10 N H_2SO_4.

Samples were taken at the beginning and at the end of the incubation period to determine the amount of VFA produced. The VFA introduced when the *P. productus* culture or sterile RACh medium was injected were taken into account when VFA production was calculated. Gas production was determined by measuring changes in gas pressure during incubation and was calculated by using the ideal gas law. The H_2 and CH_4 contents of the headspace gas were determined at the beginning and at the end of the incubation period. In a second experiment, the 100% CO_2 gas phase was replaced by an 80% H_2–20% CO_2 gas mixture. The incubation conditions, sample treatment, and analysis were the same as those used for incubation under 100% CO_2. The pressure inside the flasks at the start of the experiment was set at the ambient pressure by using either CO_2 or H_2-CO_2. The experimental results were confirmed by using ruminal samples from three other sheep.

Homofermentative character of *P. productus*. To culture tubes containing 7.4 ml of RACa medium under a 100% CO_2 headspace, 0.5 ml of a filter-sterilized, anaerobic glucose or fructose solution was added, to yield a final concentration of 50 mM. Immediately after this, 0.1 ml of a heterotrophically grown *P. productus* monoculture was added to each culture tube by using sterile syringes and needles. Incubation was performed on a rotary shaker at 37°C for 48 h. Liquid samples were taken to determine the VFA contents at 0 and 48 h by using sterile syringes and needles. CO_2 contents were determined at the end of the experiment. Controls to determine the fermentation of carbon sources brought into the culture tubes together with the *P. productus* subculture were included. The experiment was conducted in triplicate.

Radionucleotide analysis. Under a continuous CO_2 flow, 2-ml portions of sieved ruminal sample and 6.5-ml portions of CO_2-saturated, sterile Burroughs solution (pH 7.2) were added to 12 27-ml culture tubes. Before the tubes were closed with butyl rubber stoppers and crimped with aluminum seals, 0.5-ml portions of a filter-sterilized, anaerobic BES solution were added to six of the tubes, while the other tubes each received 0.5 ml of sterilized, anaerobic water. The final concentrations of BES were 0.00 and 0.03 mM. Three tubes in each group received 1 ml of a heterotrophically grown culture of *P. productus* (concentration, about $\log_{10}$ 7.00 ± 0.15 CFU/ml), while the other three tubes received 1 ml of sterile RACh medium. Finally, each culture tube received 0.5 ml of Burroughs solution containing $^{14}CH_3COOH$ (10^7 dpm). Incubations were performed on a rotary shaker for 24 h at 37°C.

After incubation, fermentation was stopped by injecting 0.2 ml of 10 M H_2SO_4. The tubes were shaken vigorously for 20 s and then left to stand for 6 h to let all of the HCO_3^- in solution evolve into CO_2 in the headspace. Five-milliliter gas samples were taken from the headspaces of the culture tubes and transferred into closed 25-ml penicillin flasks containing 10 ml of 2 M KOH under a 100% N_2 atmosphere. These flasks were shaken for 1 h at 100 rpm to trap all of the CO_2 as HCO_3^-. Then 5 ml of the liquid was transferred into 10 ml of Hionic-Fluor scintillation medium (Packard Instruments) and counted with a liquid scintillation counter (Betamatic; Kontron Instruments). Liquid samples were removed from the culture tubes at the beginning and at the end of the incubation period to extract the VFA; then the extractant (diethyl ether) was filtered through a 0.22-μm-pore-size filter, transferred into 10 ml of Hionic-Fluor scintillation medium, and counted with the liquid scintillation counter.

In the first experiment, the gas phase consisted of 100% CO_2, while in the second experiment incubation was under an 80% H_2–20% CO_2 headspace. The experiment was conducted in triplicate.

Isolation of an acetate-oxidizing, methane-producing coaggregate. Iron sulfide-reduced calcium acetate medium (44) was used in an attempt to isolate an acetate-oxidizing, methane-producing coculture. Portions (5 ml) of this medium were placed in 27-ml culture tubes under a constant 60% N_2–40% CO_2 gas flow; the tubes were closed with butyl rubber stoppers and sealed with aluminum crimps. Aliquots (100 μl) of sieved ruminal sample were injected into the culture

TABLE 1. Effect of BES on heterotrophic and autotrophic VFA production by *P. productus* ATCC 35244 during 24 h of incubation

Final BES concn (mM)	VFA production (μmol)			
	Acetic acid	Propionic acid	Butyric acid	Total
Heterotrophic production				
0	1,325 (103)[a]	43.20 (3.89)	12.60 (1.25)	1,381 (156)
0.01	1,356 (98)	42.60 (1.94)	10.90 (1.00)	1,410 (142)
0.03	1,295 (56)	45.01 (5.9)	12.30 (0.26)	1,352 (128)
Autotrophic production				
0.00	351.0 (30.7)	47.15 (2.83)	46.30 (5.78)	444.5 (33.3)
0.01	350.0 (47.4)	41.00 (5.88)	47.85 (4.20)	438.9 (43.8)
0.03	340.1 (29.0)	39.90 (3.85)	45.25 (1.90)	425.3 (34.8)

[a] The values in parentheses are standard deviations.

tubes with a syringe. The tubes were incubated without shaking at 37°C for 72 h; then 100-μl portions of the liquid cultures were transferred into fresh medium, and the preparations were incubated at 37°C for 72 h. This procedure was repeated three times. The experiments were performed three times with ruminal inocula from three different sheep, each time in triplicate.

Analysis. To determine VFA contents, samples were centrifuged at 8,000 × *g* for 20 min, and then 200 μl of each supernatant was diluted with 1.8 ml of distilled water. VFA were extracted as described previously (38) and measured with a gas chromatograph (GC) (model Di200; Delsi Instruments) equipped with a flame ionization detector, a Delsi Nermag model 31 integrator, and a capillary free fatty acid-packed column (25 m by 0.53 mm; film thickness, 1.2 μm). N_2 was used as the carrier gas at a flow rate of 20 ml/min. The column temperature was 130°C, and the temperature of the injection ports and the detector was 195°C.

The composition of the gases was analyzed with an Intersmat model IGC 120MB GC connected to a Hewlett-Packard model 3390A integrator. The GC was equipped with dual columns (an in-series connected Porapak 50- to 80-mesh column and a molecular sieve 60- to 80-mesh column) and a catharometer detector. The column temperature was isothermal at 30°C, the carrier gas was helium, and the flow rate was 10 ml/min. The percentages of N_2, CH_4, and CO_2 were determined.

The hydrogen concentration was determined by using an exhaled hydrogen monitor (GMI Medical, Ltd.) equipped with an H_2-sensitive three-electrode electrochemical cell. H_2 concentrations in the liquid phase were calculated by using Henry's law, taking into account the total gas pressure.

The gas production during batch experiments was determined by measuring the pressure in the headspace at the beginning and at the end of the incubation period and was calculated by using the ideal gas law. The total gas production was taken into account when the amounts of CH_4 and H_2 present in the headspace were determined.

Statistical analyses. To evaluate the effects of the different treatments, analysis of variance tests were performed to compare variances, after which the appropriate *t* test was used. The level of significance used was $\alpha = 0.05$. Unless indicated otherwise, the difference between two treatments was considered significant if P was < 0.05.

RESULTS

Effect of BES on heterotrophic and autotrophic growth of *P. productus* ATCC 35244 in monoculture. To investigate the possible negative effect of BES on heterotrophic and autotrophic growth of *P. productus*, BES was added at different concentrations to *P. productus* monocultures grown in RACh medium (heterotrophic) or RACa medium (autotrophic). The results of the VFA determinations at 0 and 24 h are shown in Table 1. BES had no significant influence on heterotrophic growth of *P. productus*. VFA analyses at 48 and 72 h revealed no differences in VFA production, nor were differences detected when the bacteria were counted at 0, 24, 48, and 72 h (data not shown).

The effect of BES on autotrophic growth of *P. productus* was investigated by determining the effect on its activity (i.e., reductive acetogenesis). The results of experiments to determine VFA production during incubation are shown in Table 1. Table 1 shows that addition of BES to a final concentration of 0.01 or 0.03 mM had no significant effect on either total VFA production or production of any one of the fatty acids. Spectrophotometric observations (at 590 nm) of cell growth also did not reveal differences in cell density between treatments (data not shown).

Effect of the addition of BES and/or *P. productus* ATCC 35244 on the fermentation process in ruminal samples. (i) Effect on production of VFA. A significant decrease in VFA production was observed in ruminal samples treated with BES (to which *P. productus* was not added) and incubated under a 100% CO_2 atmosphere ($P < 0.01$) (Table 2). No similar significant decrease was observed when samples were incubated under H_2-CO_2 due to higher acetate and propionate production (Table 2). Addition of *P. productus* to the ruminal samples resulted in an increase in average production of VFA, although

TABLE 2. Effect of addition of BES and/or *P. productus* ATCC 35244 on VFA production during incubation of ruminal samples for 24 h under 100% CO_2 and 80% H_2–20% CO_2

Headspace gas[a]	BES concn (mM)	*P. productus*	VFA production (mmol)			
			Acetic acid	Propionic acid	Butyric acid	Total
100% CO_2	0.00	Not added	3.91 (0.16)[b]	1.79 (0.04)	1.33 (0.03)	7.03 (0.29)
		Added	4.10 (0.20)	1.75 (0.13)	1.29 (0.07)	7.14 (0.74)
	0.01	Not added	2.64 (0.40)[c]	1.71 (0.19)	1.35 (0.11)	5.70 (0.94)
		Added	3.14 (0.22)[c]	1.66 (0.03)	1.33 (0.01)	6.13 (0.34)
	0.03	Not added	2.65 (0.21)[c]	1.76 (0.07)	1.39 (0.04)	5.80 (0.41)[d]
		Added	3.31 (0.11)[c,e]	1.76 (0.07)	1.36 (0.03)	6.43 (0.27)
80% H_2–20% CO_2	0.00	Not added	4.86 (0.41)	1.60 (0.12)	0.76 (0.05)	7.22 (0.76)
		Added	5.22 (0.37)	1.50 (0.10)	0.70 (0.07)	7.42 (0.70)
	0.01	Not added	3.76 (0.12)[c]	1.94 (0.00)[d]	1.01 (0.06)[d]	6.71 (0.12)
		Added	5.50 (0.17)[f]	1.92 (0.04)[c]	1.08 (0.03)[c]	8.50 (0.23)[f]
	0.03	Not added	3.79 (0.06)[c]	1.99 (0.03)[d]	0.95 (0.04)[d]	6.73 (0.18)
		Added	5.94 (0.18)[f]	1.92 (0.07)[c]	0.99 (0.03)[d]	8.85 (0.37)[d,f]

[a] The inocula used with the two headspace gases were different.
[b] The values in parentheses are standard deviations.
[c] Value is significantly different ($P < 0.01$) from the control value due to the addition of BES (in the presence of the same amount of *P. productus*).
[d] Value is significantly different ($P < 0.05$) from the control value due to the addition of BES (in the presence of the same amount of *P. productus*).
[e] Value is significantly different ($P < 0.05$) from the control value due to the addition of *P. productus* (in the presence of the same amount of BES).
[f] Value is significantly different ($P < 0.01$) from the control value due to the addition of *P. productus* (in the presence of the same amount of BES).

TABLE 3. Effect of addition of BES and/or *P. productus* ATCC 35244 on CH_4 production and H_2 balance during incubation of ruminal samples for 24 h under 100% CO_2 and 80% H_2–20% CO_2

Headspace gas[a]	BES concn (mM)	*P. productus*	CH_4 production (μmol)	Amt of H_2 in headspace after incubation (μmol)	H_2 concn in liquid after incubation (μM)
100% CO_2	0.00	Not added	866 (0.012)[b]	0.572 (0.034)	1.28 (0.06)
		Added	828 (0.074)	0.682 (0.073)	1.57 (0.20)
	0.01	Not added	81 (0.002)[c]	165.1 (11.6)[c]	474.2 (81.4)[c]
		Added	72 (0.002)[c,d]	45.30 (4.70)[c,e]	112.4 (10.3)[c,e]
	0.03	Not added	53 (0.004)[c,f]	160.6 (13.5)[c]	407.3 (25.7)[c]
		Added	42 (0.002)[c,e,f]	47.90 (5.40)[c,e]	121.9 (15.8)[c,e]
80% H_2–20% CO_2	0.00	Not added	1,373 (0.068)	0.350 (0.012)	1.56 (0.09)
		Added	1,020 (0.075)[e]	0.362 (0.045)	1.64 (0.14)
	0.01	Not added	58 (0.001)[c]	1,033 (34.7)[c]	5,354 (221)[c]
		Added	44 (0.005)[c,d]	47.23 (5.16)[c,e]	329.8 (32.4)[c,e]
	0.03	Not added	46 (0.003)[c,f]	1,070 (11.9)[c]	5,597 (79)[c]
		Added	42 (0.001)[c]	25.33 (1.33)[c,e,f]	216.7 (47.9)[c,e,g]

[a] The inocula used with the two headspace gases were different.
[b] The values in parentheses are standard deviations.
[c] Value is significantly different ($P < 0.01$) from the control value (no BES) due to the addition of BES (in the presence of the same amount of *P. productus*).
[d] Value is significantly different ($P < 0.05$) from the control value (no *P. productus*) due to the addition of *P. productus* (in the presence of the same amount of BES).
[e] Value is significantly different ($P < 0.01$) from the control value (no *P. productus*) due to the addition of *P. productus* (in the presence of the same amount of BES).
[f] Value is significantly different ($P < 0.01$) due to augmentation of the amount of BES (in the presence of the same amount of *P. productus*).
[g] Value is significantly different ($P < 0.05$) due to augmentation of the amount of BES (in the presence of the same amount of *P. productus*).

the effect was significant only when the preparations were incubated under an H_2-CO_2 atmosphere and contained 0.01 or 0.03 mM BES ($P < 0.01$). The increase in VFA production was attributable to greater production of acetic acid, since no significant changes in propionic or butyric acid contents were observed. Compared with BES-treated samples to which *P. productus* was not added, this greater production of acetate during incubation when *P. productus* was added to BES-treated samples coincides with a significant increase in H_2 consumption ($P < 0.01$) (Table 3). Due to the addition of *P. productus*, the H_2 concentrations in the headspaces of ruminal samples incubated under H_2-CO_2 decreased more during incubation. Also, H_2 accumulation (due to BES addition) during incubation under CO_2 was decreased by the addition of *P. productus* (Table 3). When *P. productus* was added to ruminal samples without BES, an insignificant increase in acetate production and no difference in H_2 consumption were observed during incubation under either CO_2 or H_2-CO_2.

(ii) Effect on CH_4. Addition of BES to ruminal samples incubated under CO_2 and H_2-CO_2 atmospheres caused CH_4 production to decrease in a dose-dependent way, and addition of *P. productus* to ruminal samples incubated under either CO_2 or H_2-CO_2 caused CH_4 production to decrease further (Table 3). However, the extra inhibition of CH_4 production attributable to the addition of *P. productus* was rather small and was greatest when the preparation was incubated with no BES under H_2-CO_2 (353 μmol) (significant decrease [$P < 0.01$]) or CO_2 (38 μmol) (no significant decrease).

(iii) Effect on H_2. When the control (to which *P. productus* was not added) containing the different levels of BES during incubation under CO_2 were compared, it was clear that inhibition of CH_4 production by BES caused H_2 to accumulate in the headspace (Table 3). However, the amounts of H_2 were smaller than the theoretical amount which had to be set free as determined by stoichiometric calculations (inhibition of 1 mol of CH_4 causes the release of 4 mol of H_2). This indicates that when H_2 consumption by methanogens is inhibited, other H_2-consuming processes are stimulated. These alternative H_2-consuming processes have a rather low affinity for H_2 since the H_2 concentrations in the liquid phases of BES-treated samples at the end of incubation were 474.2 and 407.3 μM in the presence of 0.01 and 0.03 mM BES, respectively (Table 3). The addition of *P. productus* to BES-treated samples incubated under CO_2 decreased the H_2 accumulation and the H_2 concentrations in the solutions significantly (112.4 and 121.9 μM in the presence of 0.01 and 0.03 mM BES, respectively [$P < 0.01$]). The H_2 threshold of *P. productus* under these incubation conditions was estimated to be 100 to 120 μM.

During incubation of ruminal samples treated with BES alone under H_2-CO_2, consumption of H_2 in the headspace was slow compared to the control. The amounts of H_2 left in the headspace were not significantly different in the presence of 0.01 and 0.03 mM BES (1,033 and 1,070 μmol, respectively [Table 3]). Since initially about 2,170 μmol of H_2 was present in the headspace and CH_4 production was very low, consumption of H_2 cannot be explained by CH_4 production. Again, the occurrence of alternative H_2-consuming processes is the basis for this difference. As in incubation under CO_2, addition of *P. productus* to the BES-treated samples caused the amount of H_2 left in the headspace at the end of the incubation period to be significantly lower (47 and 25 μmol for 0.01 and 0.03 mM BES, respectively [$P < 0.01$]). These data indicate that H_2 consumption was intense due to the addition of *P. productus.*

Table 4 provides some data about the energy gain when BES and *P. productus* were added to ruminal samples during incubation under 100% CO_2 or 80% H_2–20% CO_2. The energy gain was twofold; there was reduction of CH_4 production by BES, and there were changes (increases) in VFA production resulting from the addition of *P. productus*. During incubation under CO_2, addition of BES and *P. productus* resulted in an energy gain of about 13.1 to 15.4%; about 31.0 to 41.4% of this gain was due to the addition of *P. productus*. When BES was omitted, only a negligible energy gain (0.2%) was observed. When samples were incubated under H_2-CO_2, higher energy gains were obtained (33.5 to 36.3%), which were caused by the production of large amounts of acetate in samples to which *P. productus* was added.

Homoacetogenic character of *P. productus.* Incubation of *P. productus* with glucose or fructose (final concentration, 50 mM) revealed almost homofermentative fermentation of these carbon sources to acetate (Table 5). Although the fermentation rate for glucose was twice the fermentation rate for fruc-

TABLE 4. Energy gain after addition of BES and/or *P. productus* ATCC 35244 to ruminal samples incubated under 100% CO_2 and 80% H_2–20% CO_2

Headspace gas	BES concn (mM)	Energy gain from:				Total energy gain	
		Decreased CH_4 production due to addition of BES[a]		VFA production due to addition of *P. productus*[b]			
		kJ	%	kJ	%	kJ	%[c]
100% CO_2	0.00	0.0	0.0	18.02	100	18.02	0.2
	0.01	713.8	69.1	318.8	31.0	1,033	13.1
	0.03	740.8	59.0	513.7	41.4	1,255	15.4
80% H_2–20% CO_2	0.00	0.0	0.0	31.84	100	32	0.4
	0.01	1,195	41.8	1,651	58.0	2,846	33.5
	0.03	1,197	39.0	1,869	61.0	3,066	36.3

[a] Calculated by determining the difference in methane production between the blank and the BES-treated culture.
[b] Calculated by determining the difference in VFA concentration due to addition of *P. productus*.
[c] Calculated by determining the total energy gain on energy production by VFA production in the control (Table 2).

tose, in both cases acetate accounted for more than 99% of the total VFA production. The VFA pattern under heterotrophic conditions is therefore similar to the VFA pattern observed during heterotrophic growth of *P. productus* (Table 1). Very little CO_2 production occurred, indicating that most of the CO_2 produced during fermentation was consumed during acetate production.

Isolation of an acetate-oxidizing, methane-producing coaggregate. After three transfers into iron sulfide-reduced calcium acetate medium, no viable cells were detected microscopically. Also, no significant consumption of acetate, no CH_4 production, and no CO_2 production or consumption were observed after the second transfer. The experiments were repeated twice with inocula from different sheep, and the results were consistently negative.

$^{14}CH_3COOH$ experiment. To explain the production of large amounts of acetate when *P. productus* was added, we investigated whether utilization of acetate occurred under these incubation conditions. The decrease in $^{14}CH_3COOH$ observed during the experiment (Table 6) could have been caused either by utilization of acetate by ruminal bacteria or by oxidation of the labeled methyl group to $^{14}CO_2$. The results show that during incubation under 100% CO_2 the addition of BES and the addition of BES and *P. productus* resulted in a decrease in utilization of $^{14}CH_3COOH$ (56,500 and 58,200 dpm, respectively, compared to the control value of 76,500 dpm) (Table 6). The higher counts of $^{14}CO_2$ following addition of BES and addition of BES and *P. productus* did not reveal a higher level of oxidation of the labeled methyl group of acetate to $^{14}CO_2$, but were probably due to a lower level of reduction of $^{14}CO_2$ to $^{14}CH_4$.

Counts of $^{14}CO_2$ and $^{14}CH_3COOH$ during incubation under 80% H_2–20% CO_2 revealed that under an H_2-CO_2 atmosphere, less $^{14}CH_3COOH$ is degraded and less $^{14}CO_2$ is formed (Table 6). Degradation of labeled acetate was not significantly influenced by the addition of BES or the addition of BES and *P. productus*. When only BES was added, the $^{14}CO_2$ production was significantly different from the production of the control or from the production when BES and *P. productus* were added ($P < 0.01$). This indicates that no transformation of the methyl group of acetate to CO_2 occurred when only BES was added.

DISCUSSION

The aim of this study was to investigate if reductive acetogenesis can be stimulated in the rumen by selective inhibition of methanogenic activity, since on the basis of the results of preliminary batch incubations in which ruminal samples were used it was concluded that reductive acetogenesis did not occur when only *P. productus* was added. The inhibitor had to (i) inhibit methanogenesis selectively and (ii) have no significant effect on the heterotrophic and autotrophic growth of the reductive acetogens (in this case *P. productus*). It was found that BES did not inhibit the heterotrophic and autotrophic metabolism of *P. productus* (Table 1), which is in agreement with previous results showing selectivity of BES toward methanogens (35, 37).

That reductive acetogenesis could be stimulated by adding BES and *P. productus* was demonstrated by the decrease in H_2 concentration when a preparation was incubated under CO_2 or by the rapid utilization of H_2 during incubation under H_2-CO_2 (Table 3). The amount of H_2 after the addition of BES alone was much lower than the amount that should have occurred based on the decrease in CH_4 production. Assuming that the loss of H_2 by NO_3^- and SO_4^{2-} reduction and by utilization of H_2 for biomass is low (12), it is clear that the addition of BES alone stimulated the autochthonous reductive acetogens. Indeed, in an additional experiment selective enumeration of reductive acetogens with the enumeration procedure used to investigate the effect of BES on heterotrophic growth of *P. productus* revealed increased numbers of reductive acetogens when BES was added. The fact that reductive acetogenesis can be stimulated when H_2 accumulates has been demonstrated previously for ruminal strains (18, 21). Moreover, stimulation of reductive acetogenesis in vitro when methanogenesis is inhibited by BES has also been demonstrated to occur with washed microbial cells from swine large intestinal contents by using labeled $^{13}CO_2$ (11).

TABLE 5. Fermentation of glucose or fructose by *P. productus* ATCC 35244 in monocultures after 48 h of incubation

Substrate	VFA production (μmol)				CO_2 production (μmol)
	Acetic acid	Propionic acid	Butyric acid	Total	
Control (H_2O)	0.70 (0.18)[a]	0.04 (0.01)	0.06 (0.04)	0.800 (0.001)	0.1 (0.0)
Glucose	21.5 (2.5)	0.01 (0.01)	0.03 (0.0)	21.54 (0.010)	3.5 (0.8)
Fructose	10.8 (1.2)	0.00 (0.0)	0.01 (0.0)	10.81 (0.04)	1.5 (0.4)

[a] The values in parentheses are standard deviations.

TABLE 6. Fate of $^{14}CH_3COOH$ and production of $^{14}CO_2$ during incubation for 24 h in ruminal samples (total $^{14}CH_3COOH$ count, 10^7 dpm)

Headspace gas	BES concn (mM)	*P. productus*	Amt of $^{14}CO_2$ produced (dpm)[a]	Amt of $^{14}CH_3COOH$ removed (dpm)[b]
100% CO_2	0.00	Not added	794 (67)	79,500 (14,200)
	0.03	Not added	996 (110)[c]	56,500 (8,200)
	0.03	Added	1,055 (136)[c]	58,200 (4,600)
80% H_2–20% CO_2	0.00	Not added	678 (35)	47,700 (4,400)
	0.03	Not added	21 (33)[d]	50,000 (4,400)
	0.03	Added	658 (14)	42,500 (9,900)

[a] Total counts of $^{14}CO_2$ produced during incubation.
[b] Total counts of $^{14}CH_3COOH$ consumed during incubation.
[c] Significantly different from control ($P < 0.05$).
[d] Significantly different from control ($P < 0.01$).

At the end of the incubation of samples treated with BES and *P. productus* under CO_2, the H_2 concentration in the liquid phase varied between 100 and 120 μM (Table 3). This may represent the H_2 threshold of *P. productus*, which is about 100 times lower than the H_2 threshold of the methanogenic process (1.28 μM). This lower H_2 threshold of reductive acetogens compared with methanogens is in accordance with previous observations (19, 20, 24) and was considered the cause of the exclusive character of the hydrogenotrophic methanogens toward reductive acetogens in the human colon (20), termite cecal contents (6), and the rat colon (33). It can therefore be concluded that methanogenesis is the most active H_2-consuming process, and no other H_2-consuming processes can occur unless methanogenesis is inhibited by external manipulation. However, assuming that the difference in H_2 concentrations between BES-treated samples and samples treated with BES and *P. productus* is due to reductive acetogenesis performed by *P. productus*, the lower H_2 concentration during incubation under CO_2 or H_2-CO_2 cannot explain the higher average acetic acid production (4 mol of H_2 for 1 mol of CH_3COOH). The calculated production of acetic acid by reductive acetogenesis during incubation under 80% H_2–20% CO_2 would yield a maximum of about 0.5 mol (2 mmol of H_2 is present in the headspace), which is much lower than the values observed under these conditions (1.74 and 2.14 mmol in the presence of 0.01 and 0.03 mM BES, respectively) (Table 2).

Apart from reductive acetogenesis, other possible explanations for the production of high acetic acid levels are (i) a shift in fermentation to more oxidized components when *P. productus* was added, (ii) homofermentative growth of *P. productus*, and (iii) inhibition of acetate utilization by other bacteria (including acetate oxidation). These possibilities are addressed in detail below.

Accumulation of H_2 due to inhibition of CH_4 production or the external addition of H_2 results in a shift in fermentation to less oxidized VFA (i.e., less acetic acid) (5, 30, 35, 39). Addition of an H_2-consuming bacterium like *P. productus* lowers the H_2 concentration (Table 3) and hence shifts fermentation to more oxidized end products (acetate instead of propionate or butyrate). However, no significant change in the production of propionic acid due to the addition of *P. productus* was observed (Table 2). Also, a shift in fermentation to acetic acid would not fully explain the observed higher total VFA production compared to samples treated with BES alone.

Batch experiments with *P. productus* in which RACa medium containing glucose and fructose (50 mM) was used revealed rapid growth of *P. productus* (as determined by visual observation of turbidity) and a fermentative pattern in which acetate accounted for more then 99% of the total VFA production (Table 5), while CO_2 production was low. Therefore, *P. productus* can be considered a homoacetogenic bacterium; i.e., it produces 3 mol of acetic acid from C_6 units (14). Although degradation of the added carbon substrate was not monitored, the consumption of monosaccharides by *P. productus* might have diminished the feedback regulation of the enzymes produced by the cellulolytic or ligninolytic bacteria (4), resulting in greater degradation of these substrates and greater VFA production.

Using $^{14}CH_3COOH$, we observed that during incubation under H_2-CO_2 the difference in acetate utilization between the different treatments was at most 0.2% of the label initially present. Diminished acetate consumption therefore cannot explain the difference in acetate production observed during the batch experiment.

The hypothesis that addition of BES could have blocked acetate oxidation was examined. It has been shown that a hydrophobic coaggregate of an acetate oxidizer and a hydrogenotrophic methanogen can be present in anaerobic ecosystems (1, 44). Inhibition of methanogenesis by BES, leading to H_2 accumulation, would make acetate oxidation energetically unfavorable. The lower production of $^{14}CO_2$ during incubation under H_2-CO_2 confirmed this (Table 6). $^{14}CO_2$ production under H_2-CO_2 could therefore occur only when H_2 consumers (i.e., the methanogens in the untreated samples and *P. productus* in the sample treated with BES and *P. productus*) had diminished the H_2 pool. The absence of H_2 consumers in the sample which was treated with BES alone hampered H_2 consumption and consequently acetate oxidation, which led to almost no $^{14}CO_2$ production (Table 6). However, taking into account the concentration of $^{14}CH_3COOH$ added (10^7 dpm), we concluded that acetate oxidation was very limited. Also, several attempts to isolate a coaggregate were not successful. Due to the short retention time of the solid phase in the rumen (30 to 40 h) compared to the doubling time of the coculture (at least 30 to 40 h [1, 44]), any coculture is likely to be washed out of the rumen.

Therefore, we concluded that the higher acetate production observed when *P. productus* was added to rumen samples was probably due to the occurrence of reductive acetogenesis and homofermentative acetate production by *P. productus.*

Although it is not possible to promote the use of BES or the combination of BES and *P. productus* for commercial application in animal production due to the adaptation of the methanogens to BES (11, 23), it was demonstrated in this study that only when methanogenesis is inhibited selectively can reductive acetogenesis be promoted. For practical applications in animal nutrition, workers should search for an alternative selective inhibitor of CH_4 production compatible with acetogenesis. This inhibitor should satisfy the following requirements: (i) it should not influence heterothrophic and autotrophic growth of *P. productus* or other reductive acetogenic strains, (ii) no adaptation to it should occur, (iii) it should not harm the fermentative microbiota of the rumen (it should not inhibit VFA production), and (iv) it should not be toxic to the animal at the dose used.

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