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Influence of CH_4 Production by *Methanobacterium ruminantium* on the Fermentation of Glucose and Lactate by *Selenomonas ruminantium*

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A method is described for increasing the production of H_2 from glucose or lactate by *Selenomonas ruminantium* by sequential transfers in media containing pregrown *Methanobacterium ruminantium*. The methanogen uses the H_2 formed by the selenomonad to reduce CO_2 to CH_4. Analysis of fermentation products from glucose showed that lactate was the major product formed from glucose by *S. ruminantium* alone. Several sequential transfers in the presence of the methanogen caused a marked decrease in lactate production, which was accompanied by an increase in acetate. When lactate was the fermentation substrate, *S. ruminantium* alone produced propionate, acetate, and CO_2. Addition to the pregrown methanogen in the sequential transfer procedure caused a significant decrease in the production of propionate and an increase in acetate formed from lactate. These results are interpreted in terms of the influence of H_2 utilization by the methanogen on the production of H_2 versus lactate or propionate from reduced pyridine nucleotides by *S. ruminantium*.

Selenomonas ruminantium plays an important role in the production of volatile fatty acids, particularly propionic acid, in the rumen (3, 9). These bacteria ferment carbohydrates mainly to lactate, propionate, acetate, and CO_2 (3, 7). Some strains ferment lactate to propionate, acetate, and CO_2 (3, 7), and some produce traces of H_2 from glucose, lactate, or glycerol (8).

Physiological evidence suggests that H_2 is produced by *S. ruminantium* by oxidation of reduced pyridine nucleotides (8). Accumulation of H_2 appears to inhibit the reaction (11). Formation of H_2 is substantially increased when H_2-producing strains of *S. ruminantium* are cocultured with methanogenic bacteria that use H_2 to reduce CO_2 to CH_4 (8). Methane is produced in amounts that are significantly greater than the amount predicted from the H_2 produced by *S. ruminantium* alone.

It would be expected that as H_2 production is increased in cultures with methanogenic bacteria, electron sink fermentation products of *S. ruminantium*, i.e., lactate and propionate, would decrease because reduced pyridine nucleotide necessary for formation of these products from pyruvate would be used to produce H_2. Although previous studies showed that CH_4 formed by methanogenic bacteria increased H_2 formation by *S. ruminantium* HD4 by approximately 50-fold (8), the absolute amount of H_2 was small compared to the amount of substrate fermented. It was difficult, therefore, to detect small changes in other fermentation products. The present report describes methods for further increasing H_2 production by *S. ruminantium* during coculture with *Methanobacterium ruminantium*. These methods permit the detection of significant differences in fermentation products formed by *S. ruminantium* from glucose or lactate in the absence and presence of the methanogen. The analyses of these fermentations are included in this report.

MATERIALS AND METHODS

Organisms and growth conditions. *S. ruminantium* HD_4 and *M. ruminantium* PS were obtained from the culture collection of the Department of Dairy Science, University of Illinois. The Hungate technique as modified by Miller and Wolin (5) was used for preparation of media and cultivation. Except for routine transfers of *M. ruminantium*, the bacteria were grown in a 10-ml serum bottle containing 5 ml of a medium (SM) that contained, per liter: glucose or sodium lactate, 5.0 g; Trypticase (BBL), 5.0 g; yeast extract (Difco), 2.0 g; K_2HPO_4, KH_2PO_4, and $(NH_4)_2SO_4$, 0.24 g each; Na_2CO_3, 4.0 g; NaCl, 0.48 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.06 g; resazurin, 1.0 mg; cysteine · HCl · H_2O, 0.5 g; dithioerythritol, 15.4 mg; *n*-butyric acid, 0.58 ml; and isobutyric, 2-methylbutyric, *n*-valeric, and isovaleric acids, 0.1 ml each. The pH was adjusted to 7.0 with NaOH prior to gassing with 100% CO_2 and the addition of Na_2CO_3. The lactate-containing medium was supplemented with 10 μg each of biotin and *p*-aminobenzoic acid per liter. Glu-

cose was added aseptically to the glucose-containing medium. A 5% glucose solution was prepared in boiled and rapidly cooled water, and gassed with 100% CO_2 for 10 min. After autoclaving, the solution was cooled and added to the medium with a hypodermic syringe and needle. Routine transfer of *M. ruminantium* PS was carried out in M1 medium, which contained, per liter: Trypticase, 4.0 g; yeast extract, 2.0 g; sodium formate, 2.0 g; sodium acetate, 2.0 g; $(NH_4)_2SO_4$, 1.0 g; K_2HPO_4 and KH_2PO_4, 0.24 g each; NaCl, 0.48 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2 \cdot 2H_2O$, 0.01 g; resazurin, 1.0 mg; L-cysteine · HCl · H_2O, 0.5 g; and clarified rumen fluid, 200 ml. The medium was heated to boiling and cooled rapidly in ice while bubbling with H_2-CO_2 (50:50). Before inoculation, 30 ml of a sterile solution containing 1.25% L-cysteine · HCl · H_2O and 1.25% $Na_2S \cdot 9H_2O$, which had been adjusted to pH 10 and autoclaved under N_2, was aseptically added per liter of SM and M1 media.

For gas production and fermentation studies, 0.1 ml of a 16- to 24-h culture of *S. ruminantium* in SM-glucose was used as an inoculum. The methanogen was added (0.1 ml) from a 5- to 10-day culture in M1 medium. Several experiments involved growth of *S. ruminantium* or mixed cultures of *M. ruminantium* and the selenomonad in SM media that contained pregrown *M. ruminantium*. Pregrowth was accomplished by adding 0.1 ml of the methanogen from the M1 medium to SM medium followed by replacement of CO_2 by an H_2-CO_2 (50:50) mixture under a pressure of 2 atm and incubation for 72 h. *S. ruminantium* was added as described above, and sequential transfers were made into media with pregrown *M. ruminantium* with 0.1 ml of the mixed *S. ruminantium-M. ruminantium* culture. All gas production and fermentation studies were carried out on duplicate cultures. In some experiments, a 10-ml syringe with a 1.5-inch (ca. 3.8-cm), 22-gauge needle was aseptically inserted through the septum. Gases produced were collected in the syringe and permitted the bacterial fermentation to proceed at atmospheric pressure (5). All incubations were at 37°C.

Analytical procedures. For determination of methane and hydrogen, a 1-ml syringe fitted with a 0.5-inch (ca. 1.3-cm), 22-gauge needle was inserted through the septum and pumped at least five times before removing 0.5 ml of gas for analysis. For those cultures equipped with a syringe, the gas in the syringe was pumped back into the growth vessel several times before measuring the gas volume and removal of 0.5 ml for analysis. The gas sample was injected into a gas chromatograph (GOW-MAC Instrument Co.) with a silica gel column and a thermal conductivity detector with N_2 or Ar as the carrier gas. H_2 and CH_4 were identified and quantitated by comparison of retention times and peak heights with those of known standards.

For measurement of volatile fatty acids, cells were removed by centrifugation (13,000 × *g*) for 15 min at 5°C. A 1-ml portion of supernatant was acidified with 0.2 ml of 2 M formic acid and analyzed for volatile fatty acids (except formic acid) by gas chromatography. A stainless-steel column (6 feet by 0.125 inch [ca. 183 by 0.32 cm]) containing 6% SP-1000 on 80–100-mesh Porapak T (Supelco, Inc.) was used in a Tracor MT-150 gas chromatograph. The column temperature was 190°C, with a helium carrier gas flow rate of 120 ml/min and a flame ionization detector. The fatty acids were identified and quantitated by comparison of retention times and peak heights with those of known standards.

For other chemical analyses, the culture supernatant solutions were clarified by the Somogyi procedure (10). Glucose was assayed by the glucose oxidase procedure of the Sigma Chemical Co. (bulletin 510), except the reagent was dissolved in 0.1 M K_2HPO_4 buffer, pH 7.0. Formate was determined by the formyltetrahydrofolate synthetase method of Rabinowitz and Pricer (6) as described by Miller and Wolin (4). Ethanol was measured by the alcohol dehydrogenase method of Bonnichsen (2), and lactic acid was measured by the method of Barker and Summerson (1).

RESULTS

CH_4 was produced when *S. ruminantium* and *M. ruminantium* were simultaneously inoculated in SM-glucose medium (Table 1). The amount of CH_4 was not sufficient to cause significant changes in the products formed by *S. ruminantium* alone. Only 0.1 μmol of H_2 was produced by *S. ruminantium* alone from 139 μmol of glucose added to the medium. Increased methane production in cocultures was achieved by pregrowing *M. ruminantium* in the culture medium for 3 days with H_2-CO_2 (50:50), and then gassing with CO_2 to remove H_2, CO_2, and CH_4, and inoculating with *S. ruminantium*. After incubation for 3 days, the coculture was inoculated in medium with pregrown *M. ruminantium*. Additional transfers were made in a similar manner. Each sequential transfer produced an increase in the production of CH_4, as indicated by the increasing percentage of CH_4 in the gas phase (Table 1). Culture G (Table 1) was grown in a serum bottle with an inserted syringe to permit gas expansion to 1 atm, and a total of 71 μmol of CH_4 was produced. The amount of H_2 necessary to form CH_4 can be

TABLE 1. *Gas production by S. ruminantium and cocultures of S. ruminantium and M. ruminantium*[a]

Culture	Gas composition (%)	
	H_2	CH_4
A. *S. ruminantium*	0.1	0.0
B. *S. ruminantium* + *M. ruminantium* (simultaneous)	0.0	2.8
C. *S. ruminantium* + *M. ruminantium* (pregrown)	0.0	11.0
D. C + pregrown *M. ruminantium*	0.0	14.4
E. D + pregrown *M. ruminantium*	0.0	18.9
F. E + pregrown *M. ruminantium*	0.0	25.7
G. E + pregrown *M. ruminantium* (atm)	0.0	22.7

[a] Medium contained a total of 139 μmol of glucose.

calculated by multiplying the amount of CH_4 by 4 ($4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$). The calculated amount of H_2 produced by *S. ruminantium* in culture G was 284 μmol or 2,840 times that produced by *S. ruminantium* alone.

Products and substrate utilized were measured for *S. ruminantium* alone and also after the third transfer with pregrown *M. ruminantium* (Table 2). The predominant product formed by *S. ruminantium* alone was lactate. Small amounts of acetate and propionate were formed with traces of formate. The amount of H_2 formed was similar to that shown for *S. ruminantium* alone in Table 1 and was ignored in the calculation of the fermentation balance. With *M. ruminantium*, there was a large decrease in the amount of lactate formed by *S. ruminantium* of 88 mol/100 mol of glucose. This was accompanied by a large increase in the production of acetate by 53 mol/100 mol of glucose. Methane formed in the mixed culture was equivalent to the production of 204 mol of H_2/100 mol of glucose (4 × 51 CH_4).

Fermentation analyses were also carried out with lactate in the medium in place of glucose (Table 3). With *S. ruminantium* alone, acetate and propionate were major products. The amount of H_2 again was similar to that shown for *S. ruminantium* alone in Table 1. After three transfers with pregrown *M. ruminantium* as described above for the glucose fermentation, there was a significant alteration in fermentation products. Propionate decreased by 58 mol/100 mol of lactate, and acetate increased by 39 mol/100 mol of lactate. Methane was equivalent to the production of 156 mol of H_2 per 100 mol of lactate (4 × 39).

TABLE 2. *Fermentation of glucose by S. ruminantium without and with M. ruminantium*[a]

Product	mol/100 mol of glucose	
	S. ruminantium	*S. ruminantium* plus *M. ruminantium*
Lactate	156	68
Acetate	46	99
Propionate	27	20
Formate	4	0
Methane	0	51
Carbon dioxide	42	48
C recovery (%)	115	94
O/R	3.26	0.79

[a] Data are from the third serial transfer into the medium containing pregrown *M. ruminantium*.

TABLE 3. *Fermentation of lactate by S. ruminantium without and with M. ruminantium*[a]

Product	mol/100 mol of lactate	
	S. ruminantium	*S. ruminantium* plus *M. ruminantium*
Acetate	42	81
Propionate	73	15
Formate	2	0
Methane	0	39
Carbon dioxide	40	42
C recovery (%)	115	91
O/R	1.12	0.90

[a] Data are from the third serial transfer into the medium containing pregrown *M. ruminantium*.

DISCUSSION

Carbon recoveries and oxidation/reduction

(O/R) balances for the combined culture fermentations of glucose or lactate were reasonable (Tables 2 and 3). Carbon recoveries and O/R ratios were high for the fermentations by *S. ruminantium* alone. This may have been caused by production of small amounts of acetate by fermentation of compounds other than glucose or lactate in the medium. The acetate would lead to exceptionally high O/R values when glucose is the substrate because 1 mol of CO_2 is calculated for each mole of acetate formed, and the O/R ratio is essentially based on the small amounts of acetate, propionate, and formate produced from glucose. Endogenous substrates may have disappeared during pregrowth with *M. ruminantium*, with the result that carbon recoveries and O/R ratios differ between the fermentation by *S. ruminantium* alone and in the combined cultures.

Pregrowth of the methanogen *M. ruminantium* followed by inoculation by *S. ruminantium* and subsequent transfer of the combined cuture in media containing pregrown methanogen produces more methane than a single, simultaneous inoculation of the two organisms in a glucose-containing medium (Table 1). Similar results are obtained when lactate is substituted for glucose (unpublished experiments). Pregrowth assures that a reasonable population of methanogens is present when *S. ruminantium* initiates fermentation. Because of the rapid growth of the selenomonad and the slow growth of the methanogen, simultaneous inoculation of the separate cultures probably results in major growth of the methanogen after fermentation by *S. ruminantium* has terminated. Apparently, this does not permit sufficient interaction to cause an alteration in the products formed by *S. ruminantium*. It is not clear why continued sequential transfer of the combined-species cul-

ture on media containing pregrown methanogen causes increases in production of methane. Perhaps there are selection or induction processes favoring acetate formation by the selenomonad in the combined cultures as opposed to lactate in the single-culture fermentation of glucose and propionate in the fermentation of lactate.

The changes in fermentation products observed when the selenomonad is cultured with the methanogen are consistent with the hypothesis that H_2 is produced by *S. ruminantium* by oxidation of reduced pyridine nucleotides (PNH) and that H_2 inhibits its own production from PNH. The oxidation of PNH necessary to sustain glycolysis results in the formation of mainly lactate or propionate as electron sink products with glucose-grown (Fig. 1) or lactate-grown (Fig. 2) cells, respectively. Coculture with a methanogen removes H_2 and permits the production of H_2 from PNH to become a major electron sink. Pyruvate carbon is converted to acetate and CO_2 when pyruvate is no longer used as the substrate for production of lactate (Fig. 1) or propionate (Fig. 2). Biochemical studies, which will be reported separately, provide partial support for the hypothesis. *S. ruminantium* HD_4 does contain a very active hydrogenase despite the fact that it produces only traces of H_2. Reduction of nicotinamide adenine dinucleotide phosphate by H_2 can be demonstrated with cell-free extracts, but reduction of nicotinamide adenine dinucleotide is relatively poor. It has not yet been possible to detect the production of H_2 from either PNH. Additional studies are necessary to clarify the biochemical basis of the relationships between PNH, electron sink products, and the interaction with methanogens.

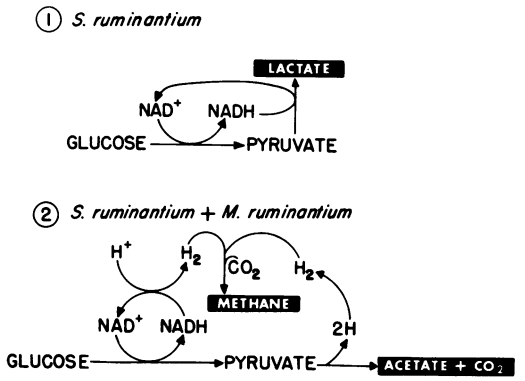

FIG. 1. *Pathway for glucose fermentation by S. ruminantium in the absence and presence of M. ruminantium.*

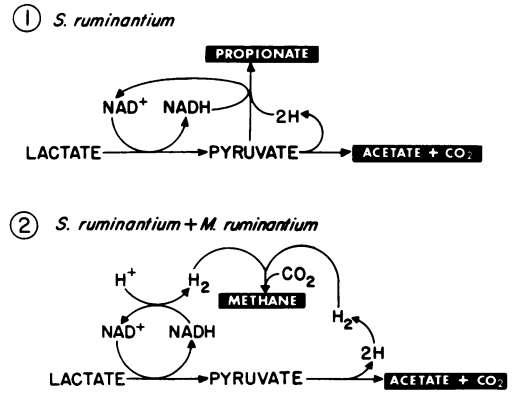

FIG. 2. *Pathway for lactate fermentation by S. ruminantium in the absence and presence of M. ruminantium.*

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