H₂ Production by Selenomonas ruminantium in the Absence and Presence of Methanogenic Bacteria

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*Selenomonas ruminantium* is a nonsporeforming anaerobe that ferments carbohydrates primarily to lactate, propionate, acetate and CO₂. H₂ production by this species has not been previously reported. We found, however, that some strains produce trace amounts of H₂ which can be detected by sensitive gas chromatographic procedures. H₂ production is increased markedly, in some cases almost 100-fold, when the selenomonads are co-cultured with methane-producing bacteria. Growth of the methane-producing bacteria depends on H₂ production by the selenomonads and the subsequent use of H₂ for the reduction of CO₂ to CH₄. Although no free H₂ accumulates in the mixed cultures, the amount of H₂ formed by the selenomonads can be calculated from the amount of methane produced. These studies indicate that the conventional methods for measuring H₂ production by pure cultures do not provide an adequate estimate of an organism’s potential for forming H₂ in an anaerobic ecosystem where H₂ is rapidly used, e.g., for formation of CH₄.

*Selenomonas ruminantium* plays an important role in the production of volatile fatty acids, particularly propionic acid, in the rumen (9). Pure cultures ferment carbohydrates mainly to lactate, propionate, acetate, and carbon dioxide (1, 9). Some strains ferment lactate to propionate, acetate, and CO₂ (1). In addition to fermenting substrates and intermediates of the mixed culture rumen fermentation to important end products, *S. ruminantium* decarboxylates succinate produced by other rumen species to propionate and CO₂ (9).

Although H₂ is produced by several species of rumen bacteria and protozoa (4) there have been no published reports of H₂ production by *S. ruminantium*. We recently found that some strains of *S. ruminantium* produce trace amounts of H₂. Formation of H₂ was substantially increased when *S. ruminantium* strains were co-cultured with methanogenic bacteria that use H₂ to reduce CO₂ to CH₄. Free H₂ did not accumulate in the combined cultures, but the increased amount of H₂ formed by *S. ruminantium* was reflected in the amount of CH₄ produced by the methanogenic bacteria. This report will be concerned with the production of H₂ by *S. ruminantium*, and the effects of methanogenic bacteria and methanogenesis on H₂ production by *S. ruminantium*.

MATERIALS AND METHODS

Organisms and growth conditions. *S. ruminantium* strains HD4, HD1, GA192, GA31, PC18, strain MOH from "Methanobacillus omelianskii" (3) and *Methanobacterium ruminantium* strain PS were obtained from the culture collection of the Department of Dairy Science, University of Illinois. *S. ruminantium* was grown with a gas phase of 100% CO₂ while the two methanogenic organisms were grown with an atmosphere of 50% CO₂/50% H₂. All stock cultures were grown on a complex medium which contained 0.1% each of glucose, cellobiose, and starch and 1.5% agar.

The basal complex medium contained per liter: Trypticase (BBL), 5.0 g; yeast extract (Difco), 1.0 g; K₂HPO₄, KH₂PO₄, and (NH₄)₂SO₄, 0.48 g each; NaCl, 0.96 g; MgSO₄·7H₂O, 0.20 g; CaCl₂·2H₂O, 0.13 g; resazurin, 1.0 mg; clarified rumen fluid, 200 ml; Na₂CO₃, 4.0 g; and cysteine-hydrochloride, 0.5 g. The basal synthetic medium contained per liter: K₂HPO₄, KH₂PO₄, and (NH₄)₂SO₄, 0.48 g each; NaCl, 0.96 g; MgSO₄·7H₂O, 0.2 g; CaCl₂·2H₂O, 0.13 g; dithiothreitol, 0.54 g; L-aspartic acid, 1.0 g; resazurin, 1.0 mg; vitamin stock, 10.0 ml; volatile fatty acid stock, 1.0 ml; Na₂CO₃, 4.0 g; and cysteine-hydrochloride, 0.5 g. The vitamin stock contained per 100 ml: thiamin-hydrochloride, calcium D-pantothenate, nicotinamide, riboflavin, pyridoxine·hydrochloride, 0.00 mg each; biotin, 0.10 mg; p-aminobenzoic acid, 1.0 mg; folic acid, 0.5 mg; and cyanocobalamin, 0.2 mg. The volatile fatty acid stock solution contained: n-butyric acid, 10.6 ml; isobutyric acid, 1.8 ml; and

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2-methylbutyric acid, n-valeric acid, isovaleric acid, 2.0 ml each. Glucose, n-lactic acid, or glycerol were added to the basal complex or synthetic media, before autoclaving, to give the final concentrations indicated in the text.

The Hungate technique for media preparation and cultivation of anaerobes as modified by Bryant (2) was used. For some experiments, the adaptation of these methods for use with serum bottles (6) was used. All incubations were at 37 C.

Gas production. For determination of methane and hydrogen, the cultures were grown in tubes (13 by 100 mm). Gas was collected by opening the tubes in a solution of 0.47 g of citric acid and 18.75 g of NaCl in 100 ml of distilled water and trapped in a graduated glass chamber fitted with a rubber septum. The total gas volume was measured by observing the displacement of liquid in the chamber (3). A 1-ml syringe fitted with 26-gauge, one-half-inch needle was inserted through the septum and pumped five times. A 0.5-ml portion of gas was removed and injected into an Aerograph gas chromatograph with a silica gel column and a thermal conductivity detector. Identification and quantitation of H2 and CH4 was based on comparison of unknowns with that of standard amounts of H2 and CH4. When serum bottles were used, a disposable syringe was inserted after the inoculation of cultures to collect and measure gas production (6). The measured gas volume in the syringe plus the measured gas space above the culture medium was the total gas volume in the system. After forcing the collected gas in and out of the serum bottle several times, the collection syringe was removed, and the gas remaining in the serum bottle was analyzed for methane and H2 by removing 0.5 ml for gas chromatographic analysis as described above.

RESULTS

H2 formation. Five strains of S. ruminantium were examined for their ability to produce H2 in a complex medium which contained 5.6 mM glucose in a total volume of 5 ml. As can be seen in Table 1, only strains HD4 and PC18 definitely produced hydrogen. The total of 0.5 μmol of H2 produced by these strains in 72 h was small in comparison to the amount of glucose added to the medium. Separate experiments showed that strains HD4 and GA192 fermented all of the added glucose in less than 24 h, and maximal growth of all five strains was achieved before 24 h. It is likely that all strains had completed the fermentation of glucose before 24 h, and the maximal amount of H2 produced per micromole of added glucose was 0.02 μmol.

H2 formation with methanogens. When one of the H2-positive strains, HD4, was co-cultured with methanogenic bacteria, either M. ruminantium PS or strain MOH, significant amounts of methane were produced (Table 2). The medium was the complex medium with glucose as the energy source and CO2 as the gas phase. Growth of the methanogens depended on growth of the selenomonad. No methane was formed by the methanogens when they were independently inoculated in the same medium without S. ruminantium, although both methanogenic strains grew and produced significant amounts of methane when the CO2 gas phase was replaced by a 50:50 mixture of H2 and CO2. Since the only substrates used by strain PS are formate or H2, and only H2 is used by strain MOH for CO2 reduction, it is clear that S. ruminantium HD4 was at least producing H2 and possibly formate to support growth of the methanogens and methane production. In the case of the mixed culture with strain MOH, where only H2 could serve as an electron donor for reduction of CO2, the total amount of H2 produced by S. ruminantium HD4 was 52 μmol. This was calculated from the total amount of methane produced in the mixed culture using the known stoichiometric relationship between H2 utilization and methane formation, i.e., 4H2

| Table 1. H2 production by strains of Selenomonas* |
|------------------------------------------|
| Strain | H2 (μmol)* |
| HD4    | 0.6        |
| HD1    | 0.1        |
| PC18   | 0.6        |
| GA192  | 0.0        |
| GA31   | 0.1        |

* Cultures were in the complex medium with 0.1% glucose in 5 ml in tubes (13 by 100 mm). Incubation was for 72 h at 37 C.

| Table 2. Formation of CH4 in combined cultures of S. ruminantium HD4 and M. ruminantium PS or strain MOH* |
|------------------------------------------|
| Organism | Initial gas phase | Total gas produced (μmol) |
|          |                  | H2 | CH4 |
| M. ruminantium PS | 50 H2:50 CO2 | 60 H2:50 CO2 | 0.0 | 12.0 (48)* |
| M. ruminantium PS or strain MOH | 100 CO2 | 0.0 | 14.2 (57) |
| S. ruminantium HD4 | 100 CO2 | 0.6 | 0.0 |
| S. ruminantium HD4 and M. ruminantium PS | 100 CO2 | 0.2 | 8.4 (34) |
| S. ruminantium HD4 and strain MOH | 100 CO2 | 0.0 | 13.0 (52) |

* Growth was for 72 h in 5.0 ml of complex medium with 5.6 mM glucose in a 10-ml serum bottle with approximately 5 ml of gas space.

* Numbers in parentheses are the H2 equivalents calculated from the amount of methane produced.
+ CO₂ → CH₄ + 2H₂O. The amount of H₂ produced in the mixed culture was, therefore, almost 100 times greater than that produced by S. ruminantium when it was cultured alone. Assuming that all of the glucose in the medium was used, the amount of H₂ produced per micromole of added glucose was 1.9 μmol.

Because strain PS can use formate as well as H₂, it is not clear which substrate was produced by S. ruminantium HD4 to support methanogenesis by strain PS. Formate production by HD4 alone was variable as determined by silicic acid chromatography and ranged from 0 mol per 100 mol of glucose to 22 mol per 100 mol of hexose when cellbiose was fermented (9). Assuming the latter ratio, formate production in the medium used for the experiment summarized in Table 2 was a total of 6.2 μmol which could theoretically support the production of 1.65 μmol of methane. The equation for methane production from formate is: 4HCO₃⁻ → CH₄ + 2H₂O + 3CO₂.

Strain HD1, which produced a trace of H₂, and strain GA192, which never showed H₂ production, were co-cultured with M. ruminantium PS in the complex medium with glucose. The results in Table 3 show that some methane was produced by the combination of HD1 and strain PS, although in this particular experiment no H₂ was detected when HD1 was cultured alone. GA192 did not produce H₂ and did not support methanogenesis by strain PS.

H₂ formation from glycerol and lactate. S. ruminantium HD4 was examined for its ability to produce H₂ when grown with glycerol or lactate instead of glucose as sources of energy.

**Table 3. Gas production in combined cultures of S. ruminantium HD1 or GA192 and M. ruminantium PS*.**

| Organism | Total gas produced (μmol) | H₂ | CH₄ |
|----------|---------------------------|----|-----|
| S. ruminantium HD1 | 0.0 | 0.0 |
| S. ruminantium GA192 | 0.0 | 0.0 |
| S. ruminantium HD1 and M. ruminantium PS | 0.0 | 3.3 (9.2)* |
| S. ruminantium GA192 and M. ruminantium PS | 0.0 | 0.0 |

* Experimental conditions were identical with those cited in Table 2 with an initial atmosphere of 100% CO₂. When M. ruminantium PS was grown simultaneously by itself in the same medium with an initial atmosphere of 50 H₂:50 CO₂, methane production was as indicated in Table 2.

* H₂ equivalents (4[CH₄]).

Production of H₂ was obtained with either substrate. When S. ruminantium HD4 was co-cultured with M. ruminantium PS with glycerol or lactate as energy sources, methane was produced, and the calculated amount of H₂ produced by S. ruminantium HD4 was significantly increased over that produced without the methanogen (Table 4). These experiments were conducted with the defined medium. No growth of S. ruminantium HD4 or S. ruminantium HD4 plus M. ruminantium PS occurred in the absence of lactate or glycerol.

**DISCUSSION**

It is not difficult to understand why the production of H₂ by S. ruminantium was not previously reported by other investigators. Unless the sensitive methods of gas chromatography are used for detection of H₂, the trace amounts produced are not apparent.

The metabolic source of H₂ is probably reduced nicotinamide adenine dinucleotide (NADH) formed during the catabolism of glucose, glycerol, or lactate. The thermodynamic barrier to the accumulation of large amounts of H₂ by oxidation of NADH was discussed previously in connection with the production of H₂ from ethanol by S organism (8) and Clostridium kluyveri (10). Formation of H₂ by S organism (3, 7) and also by Ruminococcus albus (5) was shown to be markedly increased in the presence of another species that used H₂ as a source of energy. In the cases of growth of S organism with pyruvate as an energy source (7), and R. albus with glucose as an energy source, H₂ production and subsequent utilization by a H₂-consuming species results in a marked decrease of products formed via routes that involve the reoxidation of NADH during fermentation of the energy source. S organism forms ethanol from pyruvate and R. albus forms ethanol from glucose when they are grown independently. Ethanol disappears with concomitant increases in acetate formation when either species is co-cultured with a H₂-consuming organism (5, 7).

The only organic product of carbohydrate fermentation by S. ruminantium whose formation requires NADH is propionate. Although not yet experimentally substantiated, it would be expected that increased H₂ production by S. ruminantium in the presence of a methanogen would be at the expense of propionate formation. The expression of potentially different modes of NADH oxidation, i.e., via H₂ production or propionate formation, could have significant implications with respect to the role of S. ruminantium strains in the rumen ecosystem.
TABLE 4. Gas production from lactate and glycerol by S. ruminantium HD4 in the absence and presence of methanogens

| Organism                                    | Total gas production (μmol) |
|---------------------------------------------|-----------------------------|
|                                             | No addition | 0.6% Lactate | 1% Glycerol |
|                                             | H₂       | CH₄       | H₂       | CH₄       | H₂       | CH₄       |
| Strain MOH                                  | 0.0     | 0.0       | 0.0     | 0.0       | 0.0     | 0.0       |
| M. ruminantium PS                           | 0.0     | 0.0       | 0.0     | 0.0       | 0.0     | 0.0       |
| S. ruminantium HD4                          | 0.0     | 0.0       | 0.0     | 2.5 (10)* | 0.3     | 3.2 (13)  |
| and strain MOH                              |           |           |         |           |         |           |
| S. ruminantium HD4 and M. ruminantium PS    | 0.0     | 0.0       | 0.0     | 5.3 (21)  | 0.0     | 8.3 (33)  |

*Experimental conditions were as for Table 2 with an initial atmosphere of 100% CO₂ except for the use of the defined instead of the complex medium. M. ruminantium PS and strain MOH each formed approximately 14 to 23 μmol of CH₄ when inoculated by themselves into the glycerol or lactate media with a 50 H₂:50 CO₂ atmosphere.

*S. ruminantium* appears to play a major role in the formation of propionic acid, an important product of the overall rumen fermentation (9). Recent studies in our laboratory by T. Glass showed the presence of a nicotinamide adenine dinucleotide-linked hydrogenase activity in extracts of *S. ruminantium* HD4. This supports the suggestion that H₂ produced during fermentation is a product of the oxidation of NADH and that formation of H₂ from NADH is increased when H₂ is removed by growth with a methanogen. It is not possible to completely rule out an increase in formate production by *S. ruminantium* as the cause of increased methane production when M. ruminantium PS is the added methanogen. This does not seem likely, however, because strain MOH which used H₂ but not formate for methanogenesis caused a greater flow of electrons into methane formation than did M. ruminantium PS (Table 2). In addition, it is not completely clear that *S. ruminantium* produces formate (1, 9). Small amounts of formate were reported, and all measurements involved titration of fermentation acids separated by partition chromatography (1, 9). Confirmation of formate production by more specific methods, e.g., enzymatic methods, is necessary to substantiate these reports.

The studies of H₂ formation by *S. ruminantium* and S organism (3, 7) suggest that organisms that are not classified as producers of large amounts of H₂ by conventional methods may still have the potential to produce large amounts of H₂ when H₂ is not allowed to accumulate in the environment. Co-cultivation of organisms which produce low or trace amounts of H₂ with H₂-using species, e.g., methanogens, is a useful method for indicating the physiological potential for H₂ production.

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