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Oxidation of Hydrogen and Reduction of Methanol to Methane is the Sole Energy Source for a Methanogen Isolated from Human Feces

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A methanogenic coccus isolated from human feces requires H_2 and CH_3OH for growth and uses H_2 to reduce CH_3OH to CH_4. Growth does not occur with CH_3OH alone. The organism does not grow or produce CH_4 from acetate or methylamines without or with H_2 or from H_2 and CO_2 or formate. In a complex medium, CO_2 is required for formation of approximately 50% of cell carbon, whereas the methyl carbon from methanol is not incorporated into cell carbon.

Almost all methanogens obtain energy for growth by using H_2 to reduce CO_2 to CH_4 (1). Some methanogens obtain energy by producing CH_4 from the methyl group of acetate, CH_3OH, or methylamines (1). The family *Methanosarcinaceae* contains the best known examples of methanogens that produce CH_4 from methyl groups. Conversion of CH_3OH to CH_4 proceeds according to the following equation:

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2H_2O \quad (1)$$

The electrons removed during the oxidation of 1 mol of CH_3OH to CO_2 are used to reduce 3 mol of CH_3OH to methane and H_2O.

We recently isolated a methanogenic coccus from an anaerobic enrichment of human feces in a medium that contained CH_3OH as the substrate for CH_4 production. The energy metabolism of the isolate was unique when compared to those of all previously studied methanogens. The results presented in this report show that the organism grew only when it was provided with both H_2 and CH_3OH. It used H_2 to reduce CH_3OH to CH_4 and H_2O according to the following equation:

$$H_2 + CH_3OH \rightarrow CH_4 + H_2O \quad (2)$$

The isolate did not grow on acetate or methylamines, with or without H_2, or by using H_2 to reduce CO_2 to CH_4.

MATERIALS AND METHODS

Organism. An anaerobic enrichment culture that produced CH_4 from CH_3OH had been established with an inoculum of human feces and was transferred monthly. Microscopic examination showed a large coccus, which had factor 420 fluorescence when visualized with epifluorescence microscopy, as described previously (6). Repeated attempts to isolate the coccus were unsuccessful with CH_3OH and 80% N_2–20% CO_2 (N_2-CO_2) as the gas phase. However, when the enrichment was plated on a medium which contained 0.4% CH_3OH and 101.3 kPa of 80% H_2–20% CO_2 (H_2-CO_2), a pure culture of a fluorescent CH_4-producing coccus was isolated that was morphologically similar to the fluorescent cocci in the enrichment culture. The isolate was usually in pairs or tetrads and what appeared to be clumped packets of cocci (Fig. 1). Details of the enrichment procedure and microbiological characterization of the isolate will be published separately.

Growth studies. The serum bottle modification of the Hungate technique for cultivating anaerobic bacteria was used to prepare, dispense, and autoclave media and to transfer cultures (4). All incubations were at 37°C, and liquid cultures were agitated by shaking or rolling.

The isolate was tested for its ability to grow and produce CH_4 from H_2-CO_2, formate, acetate, CH_3OH, and trimethylamine as described previously (6). Growth and CH_4 production with 0.5% methylamine, 0.5% dimethylamine, and 0.76% ethanol were examined under the same conditions.

The effect of CO_2 and H_2 on growth with CH_3OH and the stoichoimetry of CH_4 production were examined with cultures grown in a phosphate-buffered medium. It contained (per liter): Trypticase (BBL Microbiology Systems, Cockeysville, Md.) and yeast extract (Difco Laboratories, Detroit, Mich.), 2.0 g each; sodium acetate and sodium formate, 0.5 g each; thiamine-hydrochloride, nicotinamide, riboflavin, pyridoxine-hydrochloride, and calcium D-pantothenate, 2.0 mg each; cyanocobalamin, 0.2 mg; biotin, 10 mg; *p*-aminobenzoic acid, 1.0 mg; folic acid, 0.5 mg; $FeSO_4 \cdot 7H_2O$, 1.2 mg; K_2HPO_4, 0.3 g; KH_2PO_4, 2.8 g; $(NH_4)_2SO_4$, 0.3 g; NaCl, 0.61 g; $MgSO_4 \cdot 7H_2O$, 0.153 g; $CaCl_2 \cdot 2H_2O$, 76 mg; $MnSO_4 \cdot 7H_2O$, 5 mg; $CoSO_4$ and $ZnSO_4$, 1 mg each; $CuSO_4 \cdot 5H_2O$, $AlK(SO_4)_2$, H_3BO_3, and $Na_2MoO_4 \cdot 2H_2O$, 100 μg each; nitrilotriacetic acid, 15 mg; NH_4Cl, 1.0 g; L-cysteine hydrochloride, 0.875 g; $Na_2S \cdot 10\ H_2O$, 0.375 g; and resazurin, 1 mg. The pH was adjusted to 7.0 before boiling. After rapid cooling, the medium was gassed with 100%

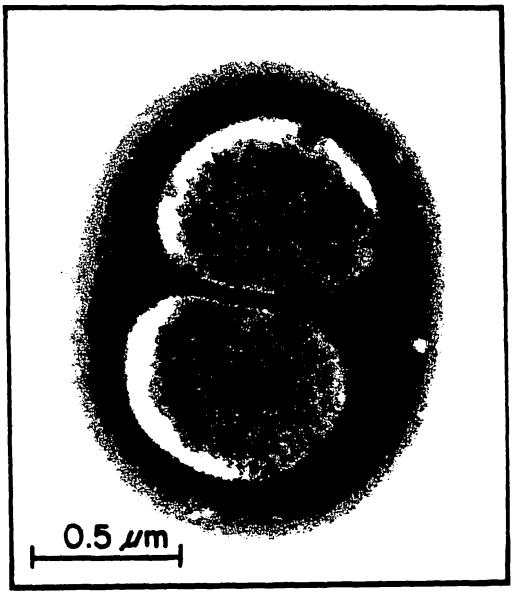

FIG. 1. Electron micrographs of cocci from a pure culture grown on 0.3% CH_3OH and 202.6 kPa of H_2-CO_2 in phosphate-buffered medium. The OD at 660 nm of the 48-h culture was 1.3. Cells on carbon- and Formvar-coated copper grids were negatively stained with a 2% aqueous solution of ammonium molybdate.

N_2. Cysteine and sulfide were added as previously described (4).

All turbidity (optical density [OD]) measurements were made with 5-ml cultures in 18- by 150-mm serum-finished tubes (Bellco Glass, Inc., Vineland, N.J.). Measurements against an uninoculated medium blank were made in a Bausch & Lomb Spectronic 70 spectrophotometer at 660 nm.

^{14}C labeling experiments. The headspaces of serum bottles of known total volume containing reduced

TABLE 1. Dependence of growth of isolate on H_2 and CH_3OH

Gas phase	OD at 660 nm with[a]	
	No addition	CH_3OH[b]
H_2-CO_2	0.27	1.90
N_2-CO_2	0.10	0.17

[a] Highest OD during 8 days of incubation. These occurred after 96 h for the H_2-CO_2 plus CH_3OH culture and after 24 h for the remaining cultures.
[b] Final concentration, 0.45% (vol/vol).

phosphate-buffered medium were gassed with H_2-CO_2 for 10 min. Then $^{14}CH_3OH$ (1.86 μCi; ca. 5,000 μmol) was added aseptically to one bottle, and cold CH_3OH (ca. 5,000 μmol) and [^{14}C]sodium bicarbonate (1.97 μCi; 0.73 μmol) were aseptically added to the other.

Each bottle was inoculated with 2.0 ml of a 48-h culture grown on phosphate-buffered medium with 0.3% CH_3OH to an OD at 660 nm of 1.1. Liquid was removed for time zero CH_3OH and radioactivity measurements. Gas samples were then removed from each bottle for H_2 measurements. (An equivalent volume of H_2-CO_2 was injected and mixed before removing gas and liquid samples.) The $^{14}CO_2$ culture was pressurized to 202.6 kPa of H_2-CO_2. The inoculated cultures were incubated for 48 to 72 h. The total gas volumes and the concentrations of CH_4 and residual H_2 were measured.

After incubation with $^{14}CH_3OH$, CO_2 and CH_4 were separated essentially by the method of Kunz (3). Briefly, 1 ml of the radioactive culture gas was mixed with cold CO_2 and CH_4 carriers and passed through a liquid N_2 cold trap to retain the CO_2. The CH_4 was passed through a gas chromatograph equipped with a molecular sieve-charcoal column and thermal conductivity detector. The CO_2 was passed through a separate chromatograph containing a silic gel column (C. Kunz, personal communication). The radioactivity of each separated gas was determined by internal gas-proportional spectrometry as described by Paperiello (7).

Five milliliters of gas was removed from each bottle and injected into serum bottles of known volume containing 4.0 ml of phenethylamine and an N_2 atmosphere. The amount of unabsorbed gas in the headspace, measured by displacement of the barrel of a hypodermic syringe, was used to calculate the amount of CO_2 absorbed. For the $^{14}CO_2$ experiment, the radioactivity of the unabsorbed gas was determined in a toluene-based scintillation fluid (11). The bottles were flushed with N_2 for 2.0 min, and the radioactivity absorbed into the phenethylamine was determined.

Incorporation of ^{14}C into cell carbon was measured by washing duplicate 6-ml portions of cell culture three times with 6 ml of distilled water. The washed cells were resuspended in 6 ml of water, and 5 ml of each was used for dry weight determinations.

The radioactivity of the resuspended cells, of CO_2 in phenethylamine, and of the liquid culture medium was determined in Aquasol (New England Nuclear Corp., Boston, Mass.) in a scintillation counter. To determine the radioactivity of the medium after incubation, the cells were first removed by centrifugation. Radioactivity of unknowns was corrected for quenching by comparing the automatic external standard ratios to those obtained with ^{14}C-quenched standards.

Other analytical methods. H_2 and CH_4 were measured by gas chromatography as previously described (2). CH_3OH was determined with a gas chromatograph with a flame ionization detector fitted with a nickel column (6 ft by ⅛ in. [ca. 182.88 by 0.318 cm]) packed with Chromosorb 101 (80/100 mesh; Supelco), under the following conditions: carrier gas, N_2 (30 ml/min); detector temperature, 155°C; column temperature, 117°C. Unknowns were quantified by comparing their retention times and peak heights with those of standards.

Chemicals. Radioactive $NaHCO_3$ and CH_3OH were obtained from New England Nuclear Corp. All gases were at least 99.999% pure. All other chemicals were of reagent grade quality or better.

RESULTS

The isolate grew and produced CH_4 from H_2 and CH_3OH, but no growth occurred when H_2 was replaced by N_2 or when CH_3OH was replaced by CO_2 (Table 1). The small amount of growth with H_2-CO_2 in the absence of methanol was probably due to the presence of small amounts of methanol in the inoculum. (The ODs of the N_2-CO_2 cultures were not significantly different from the ODs of the inoculum.) CH_4 was measured after 9 days of incubation; in cultures grown with H_2-CO_2, 30 and 3.6 μmol/ml were found in the presence and absence of CH_3OH, respectively. No growth or CH_4 forma-

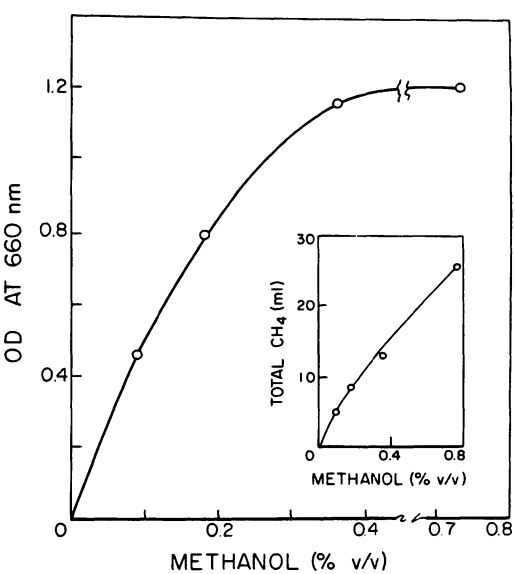

FIG. 2. Growth and CH_4 production with increasing amounts of CH_3OH. The gas phase was 202.6 kPa of H_2-CO_2. The highest OD is plotted and was attained in 2 or 3 days. All OD values were corrected for the highest value (0.20 at 48 h) of the zero methanol control. CH_4 was determined after 3 days.

tion occurred in the same medium with acetate, methylamine, dimethylamine, trimethylamine, formate, or ethanol with 101.3 kPa of H_2-CO_2 or N_2-CO_2 in the gas phase. No growth or CH_4 formation occurred with formate without or with CH_3OH with N_2-CO_2. The isolate did not grow in a complex medium which contained glucose, yeast extract, and Trypticase with 101.3 kPa of 100% CO_2.

Total growth and the amount of CH_4 formed increased as CH_3OH increased to a final concentration of approximately 0.4% with H_2 in excess (Fig. 2), or as H_2 increased to approximately 100 kPa with CH_3OH in excess (Fig. 3). Production of CH_4 continued with excess H_2 or CH_3OH after growth reached the stationary phase.

The stoichiometry of methanogenesis was determined by measuring the amounts of H_2 and CH_3OH used and the amount of CH_4 produced. Radioactive $^{14}CH_3OH$ was used to determine the incorporation of the isotope into CH_4 and CO_2. The amounts of H_2 and CH_3OH used and CH_4 produced were close to the expected values for a reaction that produces 1 mol of CH_4 from 1 mol each of CH_3OH and H_2 (Table 2). The specific activity of the CH_4 was 711 dpm/μmol, and that of CH_3OH was 670 dpm/μmol; no radioactive CO_2 was produced. The fact that CO_2 was not labeled ruled out the possibility of the formation of CH_4 according to equation (1). When the organism was grown with $^{14}CO_2$ with a final specific activity of 1,700 dpm/μmol and unlabeled CH_3OH, no radioactive CH_4 was formed. The amounts of H_2 and CH_3OH used and CH_4 formed were essentially the same for the $^{14}CO_2$ and $^{14}CH_3OH$ experiments. There was significant incorporation of the carbon of CO_2 but not that of CH_3OH into cells. Based on the specific activity of the CO_2, the amount of radioactivity incorporated, and an assumption that 50% of the dry weight of cells is carbon, we estimated that 50% of cell carbon was derived from CO_2. Separate experiments showed that CO_2 was required for growth of the organism (Fig. 4). The initial pH with 20% CO_2 was ca. 0.5 U lower than with 5% or 0% CO_2 (pH 7.1). The decrease in OD of the 20% CO_2 culture appeared to be due to lysis of the cells.

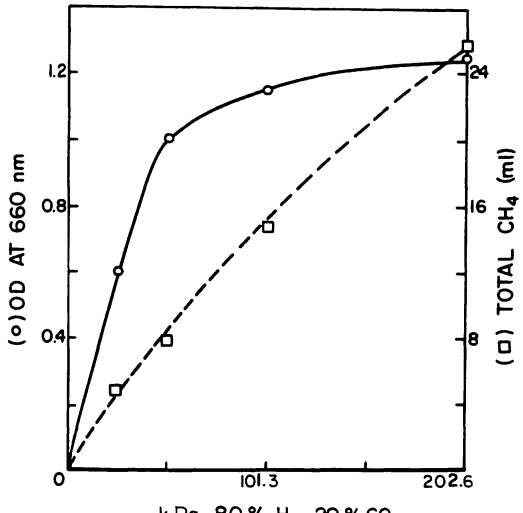

FIG. 3. Growth and CH_4 production with increasing amounts of H_2-CO_2. Methanol was 0.76%, and N_2-CO_2 was added where necessary to have all gas phases at 202.6 kPa. The highest OD is plotted and was attained at 6 to 7 days of an 8-day incubation period. CH_4 was determined after 8 days.

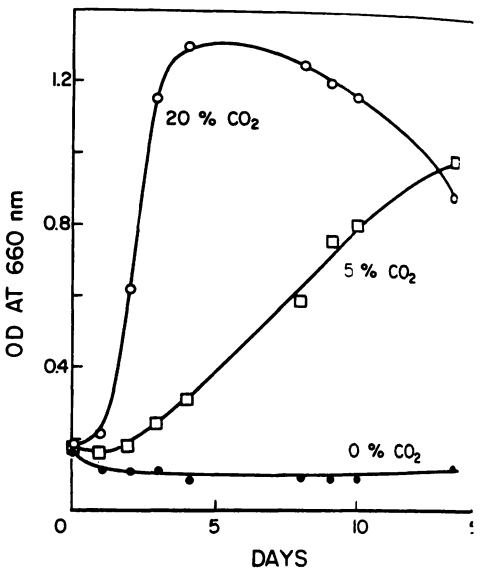

FIG. 4. Requirement for CO_2 for growth. H_2 was added to bring all gas phases to 101.3 kPa.

DISCUSSION

The organism examined in this study apparently differs from previously studied CH_3OH-using methanogens in its inability to generate electrons for the reduction of CH_3OH by oxidizing CH_3OH to CO_2. It also differs from most other methanogens in its inability to reduce CO_2 to CH_4 with H_2. Other methanogens that use CH_3OH may reduce CH_3OH to CH_4 with H_2 in addition to producing CH_4 from CH_3OH alone.

The thermodynamics of the stepwise, two-electron reductions of CO_2 to CH_4 indicates that reduction of an intermediate at the same oxidation state as CH_3OH provides almost all of the energy that can be derived from the reduction of CO_2 to CH_4 with H_2 (9). Because of its restricted

TABLE 2. Stoichiometry of production of CH_4 from CH_3OH and H_2

Compound	Total μmol		
	Found	Expected[a]	
		Equation (1)	Equation (2)
CH_3OH used	2,720	2,720	2,720
H_2 used	2,538	0	2,720
H_2 produced	2,532	2,040	2,720

[a] Based on amount of CH_3OH used.

energy metabolism, the isolate should prove useful for studies of the production of energy by the reduction of a methyl group to CH_4. The organism should also be useful for studies of biosynthesis of cell carbon from CO_2 and/or other precursors because the flow of carbon to H_2 appears to be independent of the flow from CO_2 to cell carbon.

In the intestinal habitat, the isolate probably uses for growth the CH_3OH produced by other organisms that degrade pectin (8). H_2 is a normal product or intermediate of fermentation by the human large intestine microbial community (10). The isolate was obtained from an individual who normally harbors very high concentrations of *Methanobrevibacter smithii* as the predominant methanogen (5). Although bacteria closely resembling the isolate can be identified by fluorescence microscopy in the most fecal specimens from the same individual, they are far outnumbered by *M. smithii* and are outgrown by *M. smithii* on a medium we use to enumerate predominant methanogens in the human large intestine (5). Suitable selective enumeration and isolation techniques will have to be developed to evaluate the prevalence and significance of organisms identical or similar to the isolate in the human large intestine and other methanogenic ecosystems.

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