Methanogenium frigidum sp. nov., a Psychrophilic, H₂-Using Methanogen from Ace Lake, Antarctica

PETER D. FRANZMANN,† YITAI LIU,‡ DAVID L. BALKWILL,³ HENRY C. ALDRICH,⁴ EVERLY CONWAY DE MACARIO,§ AND DAVID R. BOONE*‡

Cooperative Research Centre for the Antarctic and Southern Ocean Environment, University of Tasmania, Hobart, Australia; Department of Environmental Science and Engineering, Oregon Graduate Institute of Science & Technology, Portland, Oregon 97291; Department of Biological Science, Florida State University, Tallahassee, Florida 32306-2043; Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611; and Wadsworth Center, New York State Department of Health, Albany, New York 12201

Methanogenium frigidum sp. nov. was isolated from the perennially cold, anoxic hypolimnion of Ace Lake in the Vestfold Hills of Antarctica. The cells were psychrophilic, exhibiting most rapid growth at 15°C and no growth at temperatures above 18 to 20°C. The cells were irregular, nonmotile coccoids (diameter, 1.2 to 2.5 μm) that occurred singly and grew by CO₂ reduction using H₂ as a reductant. Formate could replace H₂, but growth was slower. Acetate, methanol, and trimethylamine were not catabolized. Cells grew with acetate as the only organic compound in the culture medium, but growth was much faster in medium also supplemented with peptides and yeast extract. The cells were slightly halophilic; good growth occurred in medium supplemented with 350 to 600 mM Na⁺, but no growth occurred with 100 or 850 mM Na⁺. The pH range for growth was 6.5 to 7.9; no growth occurred at pH 6.0 or 8.5. Growth was slow (maximum specific growth rate, 0.24 day⁻¹; doubling time, 2.9 days). This is the first report of a psychrophilic methanogen growing by CO₂ reduction.

Methanogen in cold environments is an important component of the global methane budget. Methane from wetlands is most likely the largest natural source of atmospheric methane (6, 11, 18), and about one-half of all wetlands are high-latitude wetlands (50°N to 70°N) (23). Difficulties in isolating mesophilic methanogens from these environments led to a search for obligate psychrophiles and the isolation of two psychrophilic methanogens, an aceticlastic Methanosarcina (36) and Methanococcoides burtonii (10), a methylotrophic methanogen from the hypolimnion of Ace Lake, Antarctica.

Ace Lake is a meromictic lake with a salinity level near that of seawater and with a sulfate profile that decreases with depth (sulfate becomes depleted at about 20 m) (22). Methanococcoides burtonii and many methanogens from saline, sulfate-containing habitats catabolize only methyl compounds, such as trimethylamine (10). However, in low-sulfate environments, such as the deepest parts of Ace Lake, methane production may not be restricted to noncompetitive substrates (27), such as trimethylamine. H₂ and formate may be important methanogenic substrates there.

We report here the isolation from Ace Lake of the first psychrophilic methanogen that grows on H₂ plus CO₂ or formate, and we propose the name Methanogenium frigidum sp. nov. for this organism.

MATERIALS AND METHODS

Source of inoculum. Ace Lake (68°24′S, 78°11′E) is a meromictic lake in the Vestfold Hills of Antarctica (9, 22). It is derived from marine water, and the ratios of its major cations to chloride are approximately the same as those in seawater. Salinity increases with depth, from 0.5% dissolved solids at the surface to 4.2% dissolved solids at the bottom. Sulfate also increases proportionally with depth up to a depth of 10 to 12 m, and then sulfate decreases and sulfide increases at further depths. Methane saturates the bottom waters. The sample used for inoculation of enrichment cultures was taken at a depth of 24 m (4.2% dissolved solids). The pH of the sample was 6.6, the temperature was 1.9°C, the salinity was 4.2%, and the concentrations of Mg²⁺ and K⁺ are lower. For all of the media, H₂ was added at overpressure to 200 kPa. The bottle was maintained at 4°C during transport to Antarctica, and the contents were inoculated into the medium of Jones et al. (13).

This medium was modified by decreasing the MgSO₄·7H₂O concentration to 1 g liter⁻¹, reducing the yeast extract concentration to 0.1 g liter⁻¹, omitting peptones, and adding 100 mg of vancomycin per liter. After 6 months of incubation at 8°C, faint turbidity appeared and the sample was transferred to a similar medium and incubated at 15°C. After transferring the culture several times, the pH was adjusted to 6.8.

Enrichment of methanogens. The anoxic water sample was taken to the laboratory at Davis Station, where the gas mixture was added to increase the gas overpressure to 200 kPa. The bottle was maintained at 4°C during transport to Australia, and the contents were inoculated into the medium of Jones et al. (13). This medium was modified by decreasing the MgSO₄·7H₂O concentration to 0.1 g liter⁻¹, reducing the yeast extract concentration to 0.1 g liter⁻¹, omitting peptones, and adding 100 mg of vancomycin per liter. After 6 months of incubation at 8°C, faint turbidity appeared and the sample was transferred to a similar medium and incubated at 15°C. After transferring the culture several times, the pH was adjusted to 6.8.

Growth medium and measurement of growth. Cells were routinely grown in 20 ml of liquid MSH medium in 70-ml serum bottles with butyl rubber stoppers and a gas phase consisting of N₂ and CO₂ (7:3). MSH medium (3, 25) is a bicarbonate-buffered medium with a salinity level near that of marine water. The pH of the medium (equilibrated at 15°C) was 7.2. After inoculation (1-ml inoculum), 100 kPa of H₂ was added as overpressure. During growth, a mixture of H₂ and CO₂ (4:1) was added as needed to return the total pressure to 100 kPa of overpressure. Cultures were grown on a shaker at 15°C. The pH of media equilibrated at 4°C was about 0.05 pH unit lower. To prepare media with pH values between 6.8 and 8.0, the composition of the gas was adjusted to provide an appropriate partial pressure of CO₂. For pH values below 6.8, the gas phase was 100 kPa of CO₂ and 1 M HCl was added as necessary to adjust the pH. For pH values above 8.2, the medium was flushed with N₂, and the pH was adjusted with 1 M NaOH. The pH of the culture medium tends to increase during growth of methanogens that reduce CO₂. Therefore, during growth we frequently repressurized cultures with a mixture of H₂ and CO₂ (3:1) to minimize the extent of the pH change. The pH was carefully monitored during the experiments on the effect of pH on growth, and it was always within 0.2 pH unit of the initial pH. To determine the effect of salinity on growth, we used two similar basal media, MS medium (3) is similar to MSH medium, but it does not contain added NaCl and the concentrations of Mg²⁺ and K⁺ are lower. For all of the media, H₂ was added after inoculation to a pressure of 100 kPa (above atmospheric pressure).
The inocula for experiments were always cultured in medium of the salinity being tested.

We estimated the specific growth rate ($\mu$) from the accumulated methane mass. The tritiated growth rate ($\theta$) of Methanoplanus globus fulgidus by using the equation $\frac{dM}{dt} = \frac{d\theta}{dt} = \mu M$, where $M$ is the total mass of methane produced. The relationship between $\theta$ and $\theta$ was determined using a constant growth yield ($\mu$) and by taking into account the in vivo produced methane that was not present in the vessels (28). The software TableCurve 2D, version 2.0 (AISN Software, Inc.), was used to fit the Gompertz equation to these data, which gave the maximum $\mu$ during growth of the batch culture.

The range of catalytic substrates was determined by inoculating media with various substrates and incubating the preparations for 60 days. $H_2$ was tested at a partial pressure of 1 atm, and other substrates were tested at a concentration of 200 mM. The reaction was monitored by measuring the production of controls lacking a catalytic substrate. Also, we retested substrates that did not by themselves support growth by inoculating medium with 20 mM test substrate plus 0.1 atm of $H_2$. The methane production after 60 days was compared with the methane production of controls lacking substrate and the methane production of controls containing only 0.1 atm of $H_2$. For each potential substrate tested, the cultures produced the same amount of methane from the substrate plus $H_2$ as they did from $H_2$ alone, suggesting that the potential substrate was $H_2$. 

**Influence of temperature on growth.** The effect of the temperature on the growth rate of an isolated microbe exhibits a consistent pattern. No growth occurs at temperatures at or below the minimum growth temperature ($T_{\text{min}}$), but growth occurs at temperatures above $T_{\text{min}}$. The growth rate increases with temperature up to a maximum temperature ($T_{\text{max}}$), above which growth declines. The equation has been used with a methanogen whose growth temperature range extends to the freezing point of water.

**Methane analysis.** Methane was measured by gas chromatography with flame ionization detection (20). **Antigenic fingerprinting.** Strain Ace-2 was tested with antibody S-probes prepared against reference methanogens (19) by antigenic fingerprinting (18) by using immunofluorescence and a quantitative slide immunoenzymatic assay (17). **Phylogenetic analysis.** DNA from strain Ace-2 was isolated by the chloroform-phenol method (12). A 200-bp DNA was used for a template for PCR amplification (32) of an approximately 1,400-base segment of the 16S rRNA gene. The PCR amplification primers used were p2R (ACGGTCATTCGTTACCAGCT (34) and p2F (GTCCTAGATACGGCCCTTG). The latter sequence was suggested by Carl Woese (34a) and corresponded to positions 112 to 128 in the 16S ribosomal RNA (rDNA) nucleotide sequence of Escherichia coli (6). The PCR amplification products were sequenced with an Applied Biosystems model 373A DNA sequencer by using the Taq Dye Deoxy terminator cycle sequencing method (24). The following primers were used for sequencing (34a): ACCGGGGC(G)TGTTGGCC (E. coli positions 531 to 537) and GCCCCCGC(T)CAATTCCT (positions 930 to 915). The resulting sequences were assembled to produce an approximately 734-base contiguous sequence corresponding to positions 531 to 517.

The aligned sequences were then analyzed with parsimony and distance methods. The phylogenetic analysis was performed with PHYLIP as described above. The distance matrix analysis was carried out using the PHYLIP package of microcomputer programs (7). Distances were calculated by the method of Jukes and Cantor (14), after which phylogenies were estimated with the FITCH option, in which the Fitch-Margoliash criterion (8) and some related least-squares criteria are used. **Nucleotide sequence accession number.** The 16S rDNA sequence of strain Ace-2 determined in this study has been deposited in the GenBank database under accession no. AF009219.

### RESULTS AND DISCUSSION

**Isolation of strain Ace-2**. Early attempts to isolate the dominant methanogen in agar medium were unsuccessful, so the culture was serially diluted and the highest dilution showing growth was retained for further purification. Three such successive dilutions with medium lacking antibiotics resulted in a culture devoid of visible contamination. This culture yielded no colonies when it was diluted into fluid thioglycolate medium or MSH roll tube medium lacking $H_2$. The culture was again serially diluted and inoculated into MSH liquid medium, and three successive extinction dilutions were performed to obtain a monoculture. This culture, designated strain Ace-2, was deposited in the Oregon Collection of Methanogens (Oregon Graduate Institute, Portland, Oreg.) as OCM 4691 and in the Subsurface Microbial Culture Collection, Western Branch (Oregon Graduate Institute, Portland, Oreg.), as SMCC 469W.

**Morphology.** Strain Ace-2 cells were irregular cocci 1.2 to 2.5 $\mu$m in diameter. Cells lysed immediately when they were suspended in distilled water or when 0.1 g of sodium dodecyl sulfate was added per liter of culture, suggesting that the cell wall was composed of protein (4). Cells were nonmotile. The Gram stain reaction was negative, and thin-section electron micrographs revealed an S-layer exterior to the plasma membrane (Fig. 1). The protein units appeared to be 9 to 10 nm in diameter; this value is smaller than the S-layer unit diameter of $\delta$-acaci (14 nm), but our specimens were imbedded in plastic, which often results in slight shrinkage (1). Thus, the S-layer units of strain Ace-2 and Methanogenium caraci may be the same. Exterior to the S-layer of strain Ace-2 was a fibrous coat (Fig. 1) that is absent from...
**Methanogenium cariaci** (31). No flagella or pili were found in strain Ace-2T.

**Catabolic substrates.** Cells grew with H₂ plus CO₂ as the catabolic substrate with a µ of approximately 0.24 day⁻¹ (doubling time, 2.9 days). When a 1-liter culture was provided with 1.4 liters of H₂, the culture grew to a density of about 0.5 g (wet weight) per liter. Cells grew slowly on formate (µ, approximately 0.1 day⁻¹). MSH medium contains 0.8 µM selenous acid and 0.4 µM molybdate (these metals are components of some formate dehydrogenases), so the poor growth on formate was not likely due to a lack of these metals. MSH medium also contains 0.9 µM tungstate, which at a higher concentration (1 mM) inhibits formate use by *Methanobacterium fonicicum* (11). However, growth of strain Ace-2T on formate was slow even when tungstate was omitted or when the concentrations of selenous acid and molybdate were increased. Cells did not grow on trimethylamine, methanol, or acetate. Cultures incubated with methanol plus H₂ and CO₂ produced the same amount of methane as cultures incubated with H₂ and CO₂ alone.

**Effect of temperature on µ.** The effect of temperature on the µ of strain Ace-2T was typical of bacteria in that between 0 and 20°C, µ increased with temperature to a maximum value at some point and decreased rapidly with temperature at temperatures above T_{opt} (Fig. 2). This relationship was atypical in that (i) µ was small, (ii) the temperatures for growth were low, indicating that strain Ace-2T was a psychrophile, and (iii) there was a discontinuity in µ at the freezing point of the culture medium. The last feature may logically be attributed to the transformation of water into a solid crystal matrix, which may have prevented access of the cells to H₂, prevented the detection of produced methane, or otherwise disrupted the cell metabolism. The discontinuity in µ causes the square-root equation to fit the data for strain Ace-2T poorly and to indicate that growth occurs at temperatures below freezing (Fig. 2). The fit can be improved dramatically in either of the following two ways: by restricting the range of temperatures to which the model is applied to those temperatures above the freezing point of the medium; or by modifying the equation to predict µ = 0 at temperatures below the freezing point of the culture medium (T_{freezing}), as well as at temperatures greater than T_{max}. We used the second option by applying the following equation (Fig. 2):

\[
\mu = \begin{cases} 
(1 - \exp(c - (T - T_{max})))^2, & \text{if } T_{freezing} < T < T_{max} \\
0, & \text{else} 
\end{cases}
\]

The unmodified square-root equation (29) indicated that T_{min} was -9.4°C, T_{max} was 18.1°C, b was 0.236, c was 0.0897 (for µ in units of day⁻¹), and T_{opt} was 14.9°C. The square-root equation modified with µ = 0 at temperatures at and below the freezing point of water indicated that T_{min} was -11.9°C (T_{freezing}, -1.8°C), T_{max} was 18.2°C, b was 0.224, c was 0.0849, and T_{opt} was 14.8°C.

**pH range for growth.** Cells grew well at pH values between 6.5 and 7.9 (Fig. 3). Growth at pH 8 was erratic, and cells did not grow at pH 8.5 or 6.

**Salinity.** Strain Ace-2T was a slight halophile that was unable to grow without added NaCl, although the medium contained 100 mM Na⁺ as sodium bicarbonate. Cells grew well in the presence of 300 to 600 mM Na⁺, but they could not grow in the presence of 800 mM Na⁺ or higher Na⁺ concentrations (Fig. 4). We tested growth not only in MSH medium, but also in MS medium (which differs from MSH medium in having lower concentrations of Mg²⁺ and K⁺ [3]). Growth was much faster with the higher concentrations of Mg²⁺ and K⁺ found in MSH.
FIG. 3. Effect of pH on growth. \( \mu \) was measured in MSH medium with the pH adjusted to various values.

FIG. 4. Effect of salinity on growth. \( \mu \) was measured in MSH medium with the NaCl concentration adjusted to give various Na\(^+\) concentrations. Symbols: □, medium containing 13.1 mM Mg\(^{2+}\) and 10.2 mM K\(^+\); ○, medium containing 4.9 mM Mg\(^{2+}\) and 3.5 mM K\(^+\).

FIG. 5. Phylogenetic relationship of Ace-2\(^T\) to other selected microbes, based on a distance matrix analysis of an approximately 734-base segment of 16S rRNA and rDNA sequences. The PHYLP program was used to calculate distances by the method of Jukes and Cantor, after which the FITCH option was used to estimate phylogenies from the distance matrix data. A partial tree is shown, in which Methanococcus januschii was used as the outgroup. Scale bar = 5 base substitutions per 100 bases.
ACKNOWLEDGMENTS

We thank Donna S. Williams for help with electron microscopy and Alberto J. L. Macario for assistance with the immunological work. This project was supported by grant DE-FG05-90ER61039 from the U. S. Department of Energy through the Subsurface Science Program (Deep Microbiology Subprogram) and by a subcontract from master contract 206010 (to Pacific Northwest National Laboratory), task order 258705.

REFERENCES

35. Wolfe, C. Personal communication.