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

PETER D. FRANZMANN,^{1†} YITAI LIU,² DAVID L. BALKWILL,³ HENRY C. ALDRICH,⁴
EVERLY CONWAY DE MACARIO,⁵ AND DAVID R. BOONE^{2*}

Cooperative Research Centre for the Antarctic and Southern Ocean Environment, University of Tasmania, Hobart, Australia¹; Department of Environmental Science and Engineering, Oregon Graduate Institute of Science & Technology, Portland, Oregon 97291²; Department of Biological Science, Florida State University, Tallahassee, Florida 32306-2043³; Department of Microbiology and Cell Science, University of Florida, Gainesville, Florida 32611⁴; and Wadsworth Center, New York State Department of Health, Albany, New York 12201⁵

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 by 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 peptones 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.

Methanogenesis 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* strain (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 proportionately

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 E_h was -167 mV, the total sulfide concentration was 8 mM, the sulfate concentration was 0.5 mM, and O₂ was undetectable (10). The sample was collected in a Kemmerer bottle previously rinsed with ethanol and dried. When the water was retrieved, it was immediately taken into a syringe and injected into a sterile, butyl rubber-stoppered serum bottle containing a hydrogen-carbon dioxide (4:1) gas mixture.

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 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 methane accumulated accounted for 33% of the gas. Microscopic examination revealed coccoid cells with autofluorescence typical of methanogens. The culture was transferred to fresh medium, and with incubation at 8°C turbidity recurred within 2 months. The enrichment culture was maintained by monthly transfer in a similar medium and incubation at 15°C. After transferring the culture several times, we attempted to minimize the relative numbers of nonmethanogens in the enrichment culture by including penicillin (1,000 U ml⁻¹).

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).

* Corresponding author. Mailing address: Department of Environmental Science and Engineering, Oregon Graduate Institute of Science & Technology, P.O. Box 91000, Portland, OR 97291-1000. Fax: (503) 690-1273. E-mail: boone@ese.ogi.edu.

† Present address: CSIRO Division of Water Resources, Floreat Park, WA 6014, Australia.

The inocula for experiments were always cultures grown in medium of the salinity being tested.

We estimated the specific growth rate (μ) from the accumulated methane measured periodically during growth (4) by assuming a constant growth yield and by taking into account the inoculum-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 μ during growth of the batch culture.

The range of catabolic 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 20 mM. Methane production was monitored and compared with the methane production of controls lacking a catabolic 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 substrates were not inhibitory.

Influence of temperature on growth. The effect of 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_{min}), but growth occurs at temperatures above the T_{min} , with the μ increasing with the square of the temperature (30): $\mu = b_1 \cdot (T - T_{min})^2$, where T is the temperature and b_1 is a fitting parameter. This equation only applies to temperatures between the T_{min} and temperatures well below the temperature at which growth is most rapid (the optimal temperature [T_{opt}]). The equation was later modified to conform to the decrease in μ that occurs at higher temperatures (29):

$$\mu = \left(b \cdot (T - T_{min}) \cdot \{1 - \exp[c \cdot (T - T_{max})]\} \right)^2$$

where T_{max} is the maximum growth temperature and b and c are fitting parameters. This equation describes well the relationship between temperature and μ at temperatures between T_{min} and T_{max} for a large number of bacteria (29, 37), including several methanogens (15, 16, 26). The application of this formula provides the following two major advantages: (i) a complex set of autecological data for a strain can be accurately captured and expressed by a single equation with four constants, facilitating the recording of the data in a database; and (ii) μ can be extrapolated to temperatures near T_{min} or T_{max} , temperatures at which μ is very small and therefore difficult to measure.

However, this equation applies only to growth at temperatures at or below T_{max} , whereas at temperatures above T_{max} the equation arbitrarily predicts positive μ values (37). For use with curve-fitting software, the equation to be fit to the data can be modified with an if statement to indicate $\mu = 0$ at temperatures above T_{max} (15). Alternatively, the equation itself can be modified to give negative values when $T > T_{max}$ (equation Ratkowsky 3 in reference 37). Neither 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).

Electron microscopy. Late-exponential-phase cells were fixed directly in the culture medium by mixing the culture with an equal volume of 5% unbuffered glutaraldehyde. Cells were fixed for 30 min at 4°C, collected by centrifugation, and then fixed for 30 min in cacodylate-buffered 1% osmium tetroxide. The cells were stained en bloc with 0.5% aqueous uranyl acetate and embedded in low-viscosity epoxy resin. Ultrathin sections were prepared with a diamond knife, poststained with lead citrate, and viewed with a Zeiss model EM-10CA transmission electron microscope.

Antigenic fingerprinting. Strain Ace-2^T 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^T was isolated by the chloroform-isoamyl alcohol procedure (12). Approximately 20 ng of DNA was used as a template for PCR amplification (32) of an approximately 1,400-base segment of the 16S rRNA gene. The PCR amplification primers used were rP2 (ACGGCTACCTTGTTACGACTT) (34) and GCT(G/C)AGTAACACGTGG. The latter sequence was suggested by Carl Woese (34a) and corresponded to positions 112 to 128 in the 16S ribosomal DNA (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* DyeDeoxy terminator cycle sequencing method (2, 24). The following primers were used for sequencing (34a): ACCGCGGC(G/T)GCTGGC (*E. coli* positions 531 to 517) and GCCCCG(T/C)CAATTCTT (positions 930 to 915). The resulting sequences were assembled to produce an approximately 734-base contiguous rDNA sequence corresponding to *E. coli* positions 128 to 886.

The assembled 16S rDNA sequence of strain Ace-2^T was hand aligned with the equivalent 16S rDNA or rRNA sequences of selected species belonging to the *Archaea*. The initial sets of prealigned archaeal sequences were obtained from GenBank and from the Ribosomal Database Project (21), available via the Ribosomal Database Project electronic mail server (version 5.0, updated 17 May

1995). The sequences of the following organisms were used: *Methanogenium organophilum* (GenBank accession no. M59131), *Methanogenium cariaci* (GenBank accession no. M59130), *Methanoplanus limicola* (GenBank accession no. M59143), *Methanoplanus endosymbiosus* (GenBank accession no. Z29435), *Methanomicrobium mobile* (GenBank accession no. M59142), *Methanoculleus marisnigri* (GenBank accession no. M59134), *Methanoculleus bourgenis* (RDP accession no. 33), *Methanoculleus olentangyi* (RDP accession no. 34), *Methanogenium tationis* (RDP accession no. 35), *Methanoculleus thermophilicus* (GenBank accession no. M59129), *Methanosarcina thermophila* (GenBank accession no. M59140), *Methanosaeta concilii* (GenBank accession no. X16932), *Archaeoglobus fulgidus* (GenBank accession no. Y00275, X05567), *Halococcus morrhuae* (GenBank accession no. X00662), *Thermococcus celer* (GenBank accession no. M21529), *Methanobacterium thermoautotrophicum* (GenBank accession no. X15364 and X05482), *Methanococcus jannaschii* (GenBank accession no. M59126), *Methanopyrus kandleri* (GenBank accession no. M59932), and *Thermoplasma acidophilum* (GenBank accession no. M38637 and M208022).

Each set of aligned sequences was analyzed for maximum parsimony with the program Phylogenetic Analysis Using Parsimony (33) for construction of the most-parsimonious phylogenetic tree. Only the phylogenetically informative sites were considered, and alignment gaps were retained in the analysis. A heuristic search was carried out first (with standard program defaults), after which a bootstrap analysis placed confidence limits on the branch points of the resulting phylogenetic trees. Consensus phylogenetic trees for each alignment set were produced by bootstrapping at the greater-than-50% confidence limit, with 100 replications (7). The phylogenetic position of strain Ace-2^T was inferred from its 16S rDNA sequence, as determined above, after it was aligned with sets of corresponding sequences for increasingly specific groups of archaea. The comparison sequences used for each successive alignment were selected based on the analytical results of the previous alignment. The analysis indicated that the sequence was most closely related to sequences of species of the genus *Methanogenium*. The aligned sequences were then analyzed with parsimony and distance matrix methods. The parsimony analysis was performed with PAUP as described above. The distance matrix analysis was carried out by 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^T determined in this study has been deposited in the GenBank database under accession no. AF009219.

RESULTS AND DISCUSSION

Isolation of strain Ace-2^T. 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^T, was deposited in the Oregon Collection of Methanogens (Oregon Graduate Institute, Portland, Oreg.) as OCM 469^T and in the Subsurface Microbial Culture Collection, Western Branch (Oregon Graduate Institute, Portland, Oreg.), as SMCC 469W^T.

Morphology. Strain Ace-2^T cells were irregular cocci 1.2 to 2.5 μ 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 *Methanogenium cariaci*, which is 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^T and *Methanogenium cariaci* may be the same. Exterior to the S-layer of strain Ace-2^T was a fibrous coat (Fig. 1) that is absent from

FIG. 1. Transmission electron micrographs of strain Ace-2^T. (A through C) Range of cell shapes observed. (D) Cell envelope morphology. The arrow indicates the plasma membrane, and the arrowheads indicate repeating S-layer units exterior to the plasma membrane. An electron-dense fibrous layer is visible to the left of the plasma membrane. (A and C) Bars = 1 μm. (B) Bar = 0.5 μm. (D) Bar = 0.1 μm.

Methanogenium cariaci (31). No flagella or pili were found in strain Ace-2^T.

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 formicicum* (11). However, growth of strain Ace-2^T 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-2^T was typical of bacteria in that between 0 and 20°C, μ increased with temperature to a maximum value at

FIG. 2. Effect of temperature on growth. The data points indicate values of μ measured during growth in MSH medium. The light line is the least-squares fit of the square-root equation (29), and the dark line is the best fit of the modified square-root equation (modified to predict $\mu = 0$ at temperatures below the freezing point of the culture medium).

T_{opt} 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-2^T 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-2^T 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 $\mu = 0$ 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):

$$\text{if } T_{freezing} \leq T < T_{max}, \text{ then } \mu = (b \cdot (T - T_{min})) \cdot$$

$$\{1 - \exp[c \cdot (T - T_{max})]\}^2, \text{ else } \mu = 0$$

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 $\mu = 0$ 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-2^T 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. μ was measured in MSH medium with the pH adjusted to various values.

medium, which are similar to concentrations found in seawater and in Ace Lake (10).

Requirement for growth factors. Cells grew slowly when they were inoculated into mineral medium with H_2 and CO_2 as the catabolic substrates, but after several transfers in mineral medium cells ceased to grow unless they were supplied with acetate (5 mM) as a carbon source. Thus, cells were able to grow with sulfide as the sole source of sulfur and with ammonia as the sole source of nitrogen. The presence of vitamins (35) did not stimulate growth, but growth with acetate, yeast extract, and peptones was faster than growth with acetate alone.

Antigenic fingerprinting. S-probes of antisera developed against *Methanoculleus marisnigri* JR1^T, *Methanogenium cariaci* JR1^T, the *Methanomicrobium paynteri* type strain, *Methanolobus tindarius* Tindari 3^T, *Methanoplanus limicola* M3^T and 32, and *Methanococcoides methylutens* TMA-10^T did not react with strain Ace-2^T. S-probe antibodies are prepared at a concentration that generally shows cross-reactions among strains of the same species.

Phylogenetic and taxonomic analysis. A partial 16S rDNA sequence of strain Ace-2^T was determined and compared to partial 16S rDNA sequences of other archaea. The sequence of Ace-2^T was most similar to the sequences of two *Methanogenium* species, *Methanogenium organophilum* (level of similar-

FIG. 4. Effect of salinity on growth. μ 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 PHYLIP 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 jannaschii* was used as the outgroup. Scale bar = 5 base substitutions per 100 bases.

ity, 98.6%) and *Methanogenium cariaci* (98.2%). The similarities to sequences of members of the order *Methanomicrobiales* other than *Methanogenium* strains were between 89.5 and 94.0%, and the similarities to other members of the *Archaea* that do not belong to the order *Methanomicrobiales* were 75.9 to 80.6%. A portion of the phylogenetic tree (Fig. 5) illustrates that strain Ace-2^T is most closely related to the genus *Methanogenium* and that *Methanogenium organophilum* is its closest relative. None of the other species in this genus is psychrophilic. Strain Ace-2^T is physiologically similar to previously described *Methanogenium* species, including *Methanogenium cariaci*, *Methanogenium organophilum*, *Methanogenium limicola*, and *Methanogenium tationis*. The last species is phylogenetically distinct from other members of the genus and may belong in a separate genus (5). As we found for strain Ace-2^T, all of these species are irregular cocci that grow at pH values near neutral. Also as we found for strain Ace-2^T, all grow on H_2 plus CO_2 , and all grow faster in medium with acetate and other organic growth factors; in contrast, strain Ace-2^T had a requirement for acetate. Strain Ace-2^T differed from these previously described *Methanogenium* species by being psychrophilic and by growing slowly with formate as a catabolic substrate. The phylogenetic and phenotypic data indicate that strain Ace-2^T should be classified in a new species of the genus *Methanogenium*, for which we propose the name *Methanogenium frigidum*.

Description of *Methanogenium frigidum* sp. nov. *Methanogenium frigidum* (fri'gi.dum. L. adj. [neut.] *frigidum*, cold, referring to the low growth temperatures of the species). Cells are irregular nonmotile coccoids (diameter, 1.2 to 2.5 μm) and occur singly. Pili and flagella are absent. Cells stain gram negative. Lysed by 0.1 g of sodium dodecyl sulfate per liter.

Growth is slow, and the fastest μ is 0.24 day⁻¹ (doubling time, 2.9 days). Growth is fastest on H_2 plus CO_2 ; slower growth occurs on formate. Acetate, methanol, and trimethylamine are not catabolized, but acetate is required as a carbon source. Grows in mineral medium supplemented with acetate; peptones and yeast extract are stimulatory. Sulfide can serve as

a sole sulfur source, and ammonia can serve as a sole nitrogen source.

Obligate psychrophile. The T_{opt} is 15°C, and the temperature range for growth is 17°C to the freezing point of the culture medium; no growth occurs at temperatures above 19°C. Slightly halophilic (good growth occurs in the presence of 350 to 600 mM Na⁺, but no growth occurs in the presence of 100 or 850 mM Na⁺). Fastest growth occurs at pH values between 7.5 and 7.9 (pH range for growth, 6.3 to 8.0; no growth occurs at pH 6.0 or 8.5).

The habitat is perennially cold, sulfate-depleted saline waters. The type strain is strain Ace-2 (= SMCC 459W), which was isolated from Ace Lake in Antarctica.

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