

Isolation and Characterization of a Moderately Halophilic Methanogen from a Solar Saltern

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A moderately halophilic methanogenic bacterium was enriched with trimethylamine and isolated from the sediment of a solar salt pond (total dissolved solids of pond water, 250 g/liter; pH 7.5). The isolate (strain SF1, DSM 3243) was an irregular coccus which stained gram negative, with a diameter of 1 μm and a thin monolayered cell wall. The organism grew singly, in pairs, and in irregular clumps. Colonies were tannish yellow, circular, with entire edges, and about 1 mm in diameter within 1 week. Only methylamines or methanol was used for growth and methanogenesis. Most rapid growth (doubling time, 10.2 h) occurred at a temperature of 37°C and a pH of 7.4. The optimum NaCl concentration was 2.1 M. Yeast extract or rumen fluid was required. The isolate was lysed by sodium dodecyl sulfate (0.1 g/liter) and was sensitive to chloramphenicol. The G+C content of the DNA was 41 (\pm 1) mol%.

In marine sediments where sulfate is not limiting, sulfate-reducing bacteria effectively outcompete methanogens for the major end products of anaerobic fermentation, hydrogen and acetate (17). Trimethylamine (TMA) may be the major methanogenic substrate in these environments (10, 16). TMA is produced from the breakdown of choline and betaine (4, 9, 15); choline is a component of the cell membrane of plants, and betaine is an osmolyte found in many marine plants and animals (25). TMA oxide, present in many marine animals (6), is reduced to TMA by some marine bacteria (23). In enrichment cultures from some marine sediments, TMA supports methanogenesis whereas acetate or hydrogen does not (17). Methanogenic bacteria that use only the methylamines (tri-, di-, and monomethylamine) or methanol have been isolated from such environments (11, 22, 26), and similar organisms may be present in sediments of hypersaline ponds and lakes.

Hypersaline ecosystems, though low in species diversity, may contain large numbers of microorganisms. The waters of the North Arm of the Great Salt Lake (total salinity, 332 g/liter) have up to 2.4×10^8 cells of extremely halophilic bacteria per ml, predominantly of the genera *Halobacterium* and *Halococcus*. Halotolerant, photosynthetic algae are present in concentrations of up to 10^4 cells per ml (18).

We report here the isolation and characterization of a halophilic methanogen from the sediment of a hypersaline pond. Similar organisms from the same environment were isolated by R. Paterek and P. H. Smith (Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I2, p. 140), I. M. Mathrani, D. R. Boone, and R. A. Mah (Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, I85, p. 160), and Ida K. Yu, F. Kawamura, and R. H. Doi (Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, I18, p. 149).

MATERIALS AND METHODS

Inoculum. A 100-ml sediment sample and 10 liters of water (total dissolved solids, 250 g/liter; pH 7.5) were collected from a solar saltern, pond A-22 of the Leslie Salt Co., San Francisco, Calif. The sediment was collected in a 125-ml polyethylene bottle, which was filled to the top with pond

water and tightly closed for shipment. Samples of the sediment were used to inoculate enrichment medium containing TMA.

Culture methods. The anaerobic culture techniques of Hungate (7) were used in this investigation. Stock solutions were added to culture vessels by using N_2 -flushed syringes. Experiments were done with duplicate or triplicate culture vessels. Medium was prepared by mixing all components except substrate, sulfide, carbonate, and bicarbonate and boiling for 5 min while flushing with O_2 -free N_2 . Medium was transferred to an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Mich.), dispensed into serum bottles (28 ml per 100-ml bottle), and sealed with butyl-rubber stoppers and aluminum crimp closures. If the optical density of the culture was to be measured, the medium was filtered through 1 cm of cotton before 9.2 ml was dispensed to 27-ml serum tubes. After removal from the anaerobic chamber, media were flushed for 3 min with O_2 -free gas. For solid medium, purified agar (20 g/liter) obtained from Difco Laboratories, Detroit, Mich., was added. Just before inoculation, the following were added from stock solutions as required: NaHCO_3 , Na_2CO_3 , Na_2S , and substrate (see below for medium concentrations). Media and stock solutions were autoclaved at 121°C for 20 min. All cultures were incubated at 37°C.

Media. Enrichment medium consisted of pond water with the following additions (in grams per liter): NH_4Cl , 1.0; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.40; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.10; yeast extract (Difco), 0.50; diatomaceous earth, 100; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.2; NaHCO_3 , 2.0; TMA hydrochloride, 1.91 (20 mM). The gas phase was N_2 , and the final pH was 7.3.

For isolation medium, pond water was diluted 1:1 with Milli-Q deionized water (resistivity, 17 $\text{M}\Omega \text{ Cm}$; Millipore Corp., Bedford, Mass.), with the same additions as the enrichment medium except diatomaceous earth was omitted and resazurin (1.0 mg/liter) was added. After the medium was boiled, cysteine hydrochloride monohydrate (0.50 g/liter) was added and the pH was adjusted to 7.3. The gas phase was N_2 .

After isolation, the following habitat-simulating culture medium was designed, with the major salts of seawater present at about four times the natural concentration. It was

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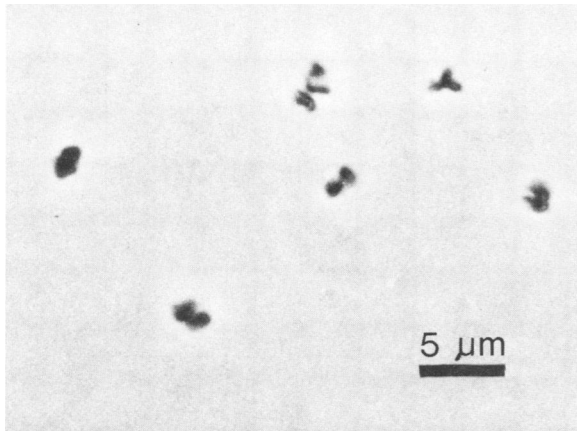


FIG. 1. Phase-contrast micrograph of mid-exponential-phase cells of strain SF1.

used in all experiments with appropriate modifications and for routine culture work. The medium consisted of the following additions to Milli-Q water (in grams per liter): NH_4Cl , 1.0; $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, 0.40; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 7.0; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 6.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.0; KCl , 3.8; NaCl , 120; yeast extract, 2.0; Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 2.0; resazurin, 0.0010; cysteine hydrochloride monohydrate, 0.50; trace minerals solution (3), 5.0 ml; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.25; Na_2CO_3 , 3.3; TMA hydrochloride, 1.91 (20 mM). The gas phase was $\text{N}_2\text{-CO}_2$ (4:1), and the final pH was 7.4.

Growth rate experiments. The growth rate constant, μ , was calculated by plotting, versus time, the natural logarithm of the sum of the accumulated methane plus methane produced by the inoculum (19).

Susceptibility to cell lysis. Lysis by sodium dodecyl sulfate was tested by adding various amounts to mid-exponential-phase cultures and comparing them to a control without additions. To test for cell lysis in hypotonic solutions, cultures were diluted with Milli-Q water and compared to a control diluted with sterile medium. Phase-contrast microscopy was used to determine lysis by comparing the numbers of cells in treated and control samples.

Sensitivity to oxygen and antibiotics. Filter-sterilized air or antibiotics were added to mid-exponential-phase cultures. The culture vessels containing oxygen and the appropriate controls were incubated in a shaker. Methane production was measured and compared to that in controls with O_2 -free N_2 or anaerobic water added. Sensitive cultures were transferred (3.3% inoculum) to fresh medium to test for cell viability.

G+C content. Exponential-phase cells were lysed with sodium dodecyl sulfate, and the DNA was purified according to the technique of Marmur (14). The buoyant density of the purified DNA was measured by ultracentrifugation in a CsCl density gradient (20), and the base ratio (in moles percent of G+C) was calculated by using the formula of Schildkraut et al. (21).

Electron microscopy. Late-exponential-phase cells were prefixed with 4% glutaraldehyde, washed with 12% NaCl , and fixed by the method of Kellenberger et al. (8). The fixed sample was embedded in epoxy-araldite resin, thin sectioned, and poststained with uranyl acetate and lead citrate. The electron microscope used was a Zeiss 109.

Analytical techniques. Methane was measured by gas

chromatography (1). Optical density was measured at 570 nm with a path length of ca. 1.6 cm.

RESULTS

Enrichment and isolation. Enrichment and isolation were performed by Robert A. Mah. Sediment was inoculated (5%, vol/vol) into enrichment medium containing 20 mM TMA. Methane was detected in the cultures after 3 days, and within 15 days all substrate was used. More TMA (20 mM) was added and used within 7 days.

The enrichment culture was serially diluted in isolation medium and used to inoculate roll tubes. Small (less than 1 mm in diameter) fluorescent colonies were observed in roll tubes inoculated with the equivalent of 5 μl of enrichment culture, and methane was detected in these tubes. Colonies were picked and inoculated into fresh roll-tube medium. This procedure was repeated until only one colony type was observed. The isolate was named strain SF1 (DSM 3243).

Colony and cellular morphology. Surface colonies of the isolate were tannish yellow, with diameters of 1 mm within 1 week, convex, smooth, and shiny, with entire edges and a grainy interior. Subsurface colonies had the same morphology and color and were lenticular. Under epifluorescence microscopy, colonies were bright blue and did not fade. Cells in exponential phase were refractile, irregular cocci, about 1 μm in diameter, occurring singly, in pairs, and in small irregular clumps (Fig. 1). Exponential-phase cells stained gram-negative. Under epifluorescence microscopy the cells were bright blue and faded after 1 min. A thin cell wall was visible in transmission electron micrographs (Fig. 2); flagella or pili were not observed.

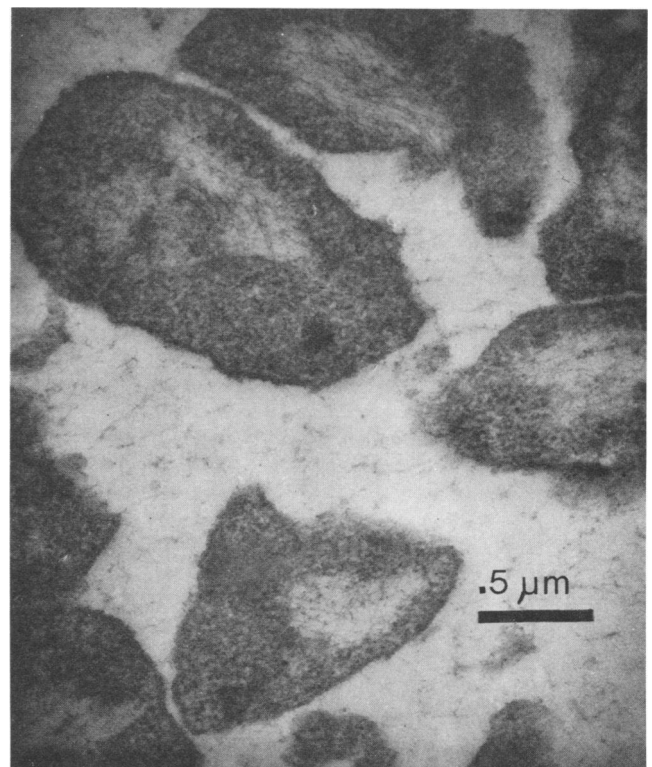


FIG. 2. Transmission electron micrograph of a thin section of strain SF1.

Sodium dodecyl sulfate (0.1 g/liter) lysed mid-exponential-phase cells. Lysis in hypotonic solution was also demonstrated. Diluting cell suspensions to 1:3 with water caused swelling of the cells, and diluting to 1:10 caused complete lysis.

The antigenic cross-reactivity of the isolate with fluorescent rabbit antibody raised against other strains of methanogenic bacteria was tested by E. Conway de Macario (2, 13). The isolate scored zero with the 25 S-probe antisera. However, a slight reaction (below the level considered 1) was noted with the *Methanosarcina barkeri* R1M3 and *Methanosarcina mazei* MC6 (strain S-6) probes.

Growth and physiology. Methane and optical density increased at the same exponential rate during growth. Acetate, TMA, dimethylamine, monomethylamine, methanol, formate (all at 20 mM) and H_2 - CO_2 (4:1, 50 kPa overpressure) were tested as methanogenic substrates, alone and with H_2 present. Only TMA, dimethylamine, monomethylamine, and methanol supported growth. The methylamines and methanol were converted stoichiometrically to methane. During growth on methylamines or methanol, added H_2 had no effect on the final amount or rate of methane production.

Yeast extract or rumen fluid was required for optimal growth. Trypticase peptone and sludge supernatant fluid were stimulatory but alone would not support growth. The optimum growth temperature was near 37°C, with a maximum growth temperature between 40 and 45°C (Fig. 3). The optimum pH for growth was about 7.4, with growth occurring from pH 6.8 to 8.3 in a bicarbonate- CO_2 buffer (Fig. 4). The optimum NaCl concentration was about 2.1 M, and the isolate grew well from 0.86 to 3.4 M (Fig. 5). KCl could not replace NaCl. If magnesium and calcium were omitted no growth occurred (0.3% [vol/vol] inoculum). Either magnesium (57 mM) or calcium (56 mM) alone supported optimum growth. A requirement for the omitted cation may have been met by trace impurities in the added cation (a maximum of 1 mM calcium when magnesium was the only divalent cation added or 3 mM magnesium when calcium was added). The minimum observed doubling time was 10.2 h ($\mu = 0.068/h$). The G+C content of the DNA was 41 (± 1) mol%. Liquid cultures stored at room temperature for 6 months were viable and fluoresced brightly, but the fluorescence faded quickly.

The isolate was sensitive to chloramphenicol and insensitive to penicillin, carbenicillin, cycloserine, erythromycin,

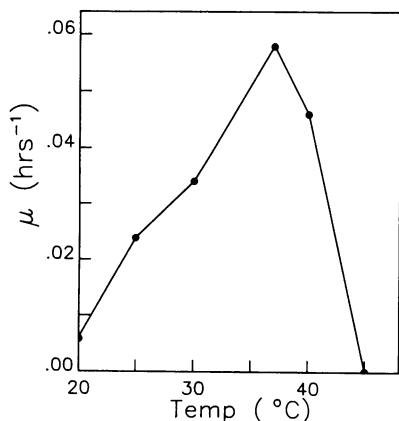


FIG. 3. The effect of temperature on the growth rate of strain SF1.

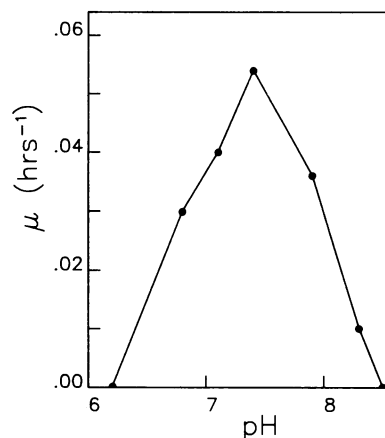


FIG. 4. The effect of pH on the growth rate of strain SF1.

and tetracycline (all at 100 μ g/ml). The chloramphenicol-inhibited cultures grew when inoculated into fresh medium without antibiotic. When air was added to culture vessels (4.4% final O_2 content), methanogenesis and growth were completely inhibited, but cells remained viable even after 1 week of exposure.

DISCUSSION

Strain SF1 was similar to three previously reported methanogens, *Methanobrevibacter tindarius* (11), *Methanococcoides methylutens* (22), and *Methanococcus halophilus* (26). These three strains were isolated from marine sediments. They are slightly halophilic, irregular cocci that use only the methylamines or methanol for growth. Strain SF1 and these isolates all have thin cell walls that stain gram negative and are sensitive to lysis by sodium dodecyl sulfate. These four organisms represent a distinct physiological group within the methanogenic bacteria: the halophilic, methylotrophic, nonacetate-utilizing methanogens.

Strain SF1 differed from the other strains in several ways. The salt requirement of strain SF1 was much higher than that of the other methanogens. The requirement for 2.1 M NaCl for optimum growth places strain SF1 among the moderate halophiles (12). The maximum concentration required among the three previously described halophilic methylotrophs is 1.2 M NaCl for *Methanococcus halophilus*

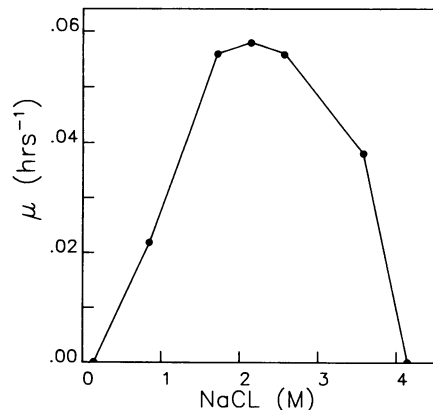


FIG. 5. The effect of NaCl concentration on the growth rate of strain SF1.

(26). This characteristic reflects the differences in the habitats from which SF1 and the other strains were isolated.

The optimum growth conditions of strain SF1 and the other methylotrophs also differed with respect to temperature and pH. Strain SF1 had a slightly higher temperature optimum than any of the other three bacteria, whose optimal growth temperatures range from 25 to 36°C. The optimum pH for growth of the other halophilic methylotrophs ranges from 6.5 to 7.5. SF1 had an optimum (pH 7.4) at the upper end of this range and grew at a pH higher than that reported for the other halophilic, methylotrophic methanogens.

The extreme halophiles and the methanogenic bacteria are both members of the proposed procaryotic urkingdom, the *Archaeobacteria* (24). In fact, the methanogens and the halophiles are more closely related to each other than to the third group within the *Archaeobacteria*, the thermoacidophiles (5). Whether strain SF1 is more related to the methanogens or is an evolutionary intermediate between the methanogens and the halophiles is not known.

ACKNOWLEDGMENTS

We thank Robert A. Mah for sample collection and for performing the enrichment and isolation. We also thank Michael Smith and Paul H. Smith for sample collection, Jack Pangborn, Facility for Advanced Instrumentation, University of California, Davis, for preparing the electron micrographs, Everly Conway de Macario, Center for Laboratories and Research, New York State Health Department, Albany, N.Y., for the antigenic serotyping of the isolate, and Robert Sleat, Tom Ferguson, Yitai Liu, Bernard Ollivier, Mario Panaqua, and Harvey Negoro for helpful discussions.

This research was supported by a grant from the U.S. Department of Energy (DE-AT03-80ER10684).

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