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Isolation and Characterization of a Halophilic Methanogen from Great Salt Lake†

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A halophilic methanogenic microorganism isolated from sediments collected from the southern arm of Great Salt Lake, Utah, is described. Cells were irregular, nonmotile cocci approximately 1.0 μm in diameter and stained gram negative. Colonies from anaerobic plates and roll tubes were foamy, circular, and cream-yellow. Methanol, methylamine, dimethylamine, and trimethylamine supported growth and methanogenesis. Hydrogen-carbon dioxide, formate, and acetate were not utilized. Sodium and magnesium were required for growth; the optimum NaCl concentration ranged between 1.0 and 2.0 M, with the minimum doubling time occurring at 2.0 M. The optimum growth temperature was 35°C, with maximum growth rate occurring at pH 7.5. The DNA base composition was 48.5 mol% guanine + cytosine. SLP is the type strain designation (= ATCC 35705).

Although methanogenic bacteria and the extreme halophilic bacteria are both members of the kingdom *Archaeobacteria* (22), there is little known about methanogens or methanogenesis in hypersaline ecosystems. However, some general observations of methanogenesis in such ecosystems have been reported. Inocula of sediments and water samples from the northern arm of the Great Salt Lake produce methane in vitro (21; D. M. Ward, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, I49, p. 89), and in situ methane production also occurs (14). Furthermore, formaldehyde inhibits this reaction during in vitro incubation, indicating a biological origin for methanogenesis (Ward, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978). The methyl groups of methylmercaptan and methionine were also reported as possible substrates for methanogenesis in this lake (T. Phelps and J. G. Zeikus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, I4, p. 85).

Biogenic methane from hypersaline environments has been reported; in the Gulf of Mexico a submarine brine pool with a salinity of 188 g/liter has a methane content of 2.0 ml/liter (3). Methanol, trimethylamine, and methionine enrichments from Big Soda Lake, an alkaline, moderately hypersaline desert lake (pH 9.7 and salinosity of 87 g/liter) also produced methane. Hydrogen, acetate, and formate stimulated methanogenesis slightly or not at all in these enrichment cultures (13).

We examined the sediments of the northern and southern arms of Great Salt Lake for the presence of methanogenic bacteria. These bacteria are of interest because they are involved in the terminal dissimilation of organic carbon in nutrient-rich hypersaline environments. These microorganisms may also be involved in nitrogen metabolism by their utilization of methylamines (10). These amines, primarily trimethylamine, may be formed from phosphatidylcholine via choline, as reported to occur in the rumen (12). Betaine and trimethylamine oxide, both osmolytes produced in a number of plants and animals exposed to hypersaline environments (24), may also be converted by microorganisms in these ecosystems to trimethylamine (J. R. Paterek and P. H. Smith, manuscript in preparation). Biological importance of halophilic methanogenic bacteria is also implicated by a phylogenetic relationship which might bridge between the families *Halobacteriaceae* and *Methanobacteriaceae*.

Portions of these results were presented previously (J. R. Paterek and P. H. Smith, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I2, p. 140).

MATERIALS AND METHODS

Site description and sampling. The Great Salt Lake in northern Utah is a thalassohaline terminal lake that is divided by a rock and gravel railroad causeway into northern and southern arms. These arms differ in general limnological properties (17, 21; Phelps and Zeikus, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980). Samples were collected from both the northern and southern arms of this lake.

A plexiglass tube (3.0 cm by 1.0 m) was used to collect sediment. Subcores taken with a sterile no. 6 brass cork borer were transferred to sterile 50-ml serum bottles which were filled to capacity with the sample. These bottles were sealed with butyl rubber stoppers held in place with aluminum crimps. Samples were transported to our laboratory on ice and processed within 48 h of collection.

Isolation procedure and media. All media utilized in this study were prepared according to the technique of Hungate (7) with modifications (2, 4). Isolation medium contained (% [wt/vol]): ammonium chloride, 0.05; yeast extract (Difco Laboratories, Detroit, Mich.), 0.1; Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 0.2; CoM (sodium 2-mercaptoethanesulfonate), 0.0001; sodium acetate, 0.05; sodium formate, 0.05; DL-methionine, 0.05; methanol, 0.05; sodium carbonate, 0.1; sodium bicarbonate, 0.2; sodium sulfide nonahydrate, 0.05; cysteine hydrochloride, 0.05; trace minerals solution (23), 1.0; vitamins solution (23), 1.0; volatile fatty acids solution (16), 1.0. The liquid portion of this medium was filtered brine (GF/D glass microfibre filter; Whatman Inc., Clifton, N.J.) collected at the respective sampling sites of Great Salt Lake. Thus, each sediment sample was cultivated in medium at the salinity of its ecosystem. The gas phase was hydrogen-carbon dioxide (80 and 20%, respectively) at a pressure of 170 kPa. Before being autoclaved, the pH was adjusted with 2.0 N KOH to give a final value of pH 7.2.

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Serial dilutions (1:10) of the sediments were prepared in 9.0 ml of sterile medium. Triplicate agar roll tubes (2% Noble agar; Difco) were inoculated from each dilution. After 8 weeks of incubation at 30°C, isolated colonies were selected from the highest dilution that produced methane. These colonies were picked and again serially diluted in agar roll tubes as described. The procedure was repeated until only one type of colonial morphology was obtained. The purity of the microorganism exhibiting this morphology was assured by examination of wet mounts of the isolate both by phase-contrast and epifluorescence microscopy (11). Representative cultures of the isolate were inoculated into basal medium and supplemented with glucose (0.05% [wt/vol]). A selective inhibitor of methanogens, BES (2-bromoethanesulfonic acid), was added at a concentration of 2×10^{-2} M to the glucose media to favor growth of contaminating microorganisms. Such cultures were incubated both aerobically and anaerobically.

The following medium was used for growth rate and methane productivity measurements (all experiments were done in triplicate unless noted). Components in final concentrations (% [wt/vol]): NH_4Cl, 0.05; yeast extract, 0.1; Casamino Acids (Difco), 0.1; Trypticase peptone, 0.1; cysteine hydrochloride, 0.05; trace minerals solution (23), 1.0; vitamins solution (23), 1.0; volatile fatty acids solution (16), 1.0; sodium bicarbonate, 0.2; sodium carbonate, 0.2; sodium sulfide, 0.05; substrate (usually trimethylamine hydrochloride), 0.25. The above medium was prepared in the following brine-containing salt concentrations approximating that of the southern arm of Great Salt Lake (14) (percent [wt/vol]); NaCl, 8.07; $MgCl_2 \cdot 6H_2O$, 3.51; KCl, 0.57; $CaCl_2$, 0.055; LiCl, 0.013; H_3BO_3, 0.012; and Na_2SO_4, 1.29. The medium was brought to volume with glass-distilled water. The medium had a gas phase of 80% N_2/20% CO_2 and a final pH of 7.4. This medium was utilized in this study unless otherwise noted.

Substrate determinations were made and antibiotic sensitivities were determined by introducing a 5% inoculum of the isolate. These determinations utilized 10 ml of medium in Balch serum tubes (18 by 15 mm; Bellco Glass Co., Vineland, N.J.) sealed with butyl rubber stoppers. Tubes were incubated without shaking at 35°C. Growth was followed by measuring optical density at 610 nm and by measuring methane production.

Temperature growth profiles were incubated without shaking for 2 weeks before methane was determined. KOH (8 N) and HCl (5 N) were added to growth medium to establish a range of pH values. The sodium chloride concentration was also varied to determine the optimum concentration for the isolate.

Gas analysis. Methane gas was quantified by using gas chromatography with thermal-conductivity detection. Hyperbaric gas pressures in the culture tubes were measured with a digital pressure transducer and indicator (Setra Systems, Acton, Mass.).

Microscopy and photomicroscopy. Phase-contrast photomicrographs were prepared from wet mounts on slides which were coated with a 1.0% Noble agar and air dried.

Electron microscopy. Cells at mid-logarithmic growth phase were fixed in 2% glutaraldehyde–2% osmic acid made up in 4.0 M NaCl. After 1.5 h of fixation, cells were washed three times with pH 7.2 cacodylate buffer (0.2%). The final cell pellet was solidified in agar, dehydrated with ethanol, and embedded in the low viscosity medium described by Spurr, (20). Thin sections were prepared with an Ultramicrotome III (LKB Instruments Inc., Rockville, Md.). Uranyl acetate and lead citrate were used as post stains, and electron micrographs were taken with a JEOL 100 CX electron microscope.

Mole percent of guanine plus cytosine. Cells were lysed by suspending a centrifuged (5,000 × *g*, 30 min) pellet in 5% (vol/vol) Triton X-100, and the DNA was purified (9). Buoyant density of the DNA was determined by centrifugation (35,000 × *g*, 40 h) in a CsCl density gradient containing acrylamide polymerization reagents (15). The relative positions of the test DNA and DNA from five standards were found by staining the acrylamide gels with ethidium bromide and measuring fluorescence at 254 nm with an Aminco fluoro-colorimeter. The method of Schildkraut et al. (18) was used to calculate the base ratio.

RESULTS

Small (<3 mm), cream-yellow, circular colonies that showed yellow-green fluorescence (420-nm excitation wavelength) appeared in agar roll cultures of sediment samples from the south arm of Great Salt Lake (24% NaCl saturation). This colony type was the predominant fluorescent form in all culture tubes examined. Isolated colonies were picked and recultured in the isolation medium until one colony morphology occurred. The purity of the isolate was supported by the lack of growth in complex broth media when inoculated under nonmethanogenic conditions. A single cell morphology was observed under phase-contrast and epifluorescence (420 nm) microscopy. The isolate was named strain SLP (Salt-Lake-Paterek).

Cells were irregular cocci with a diameter of 0.8 to 1.8 μm (Fig. 1 and Fig. 2) and forming clumps in older cultures (data not shown). Electron photomicrographs (Fig. 3) revealed a defined membrane and cell wall. The cells stained gram negative and were nonmotile in anaerobic hanging-drop preparations. No flagella were seen in electron photomicro-

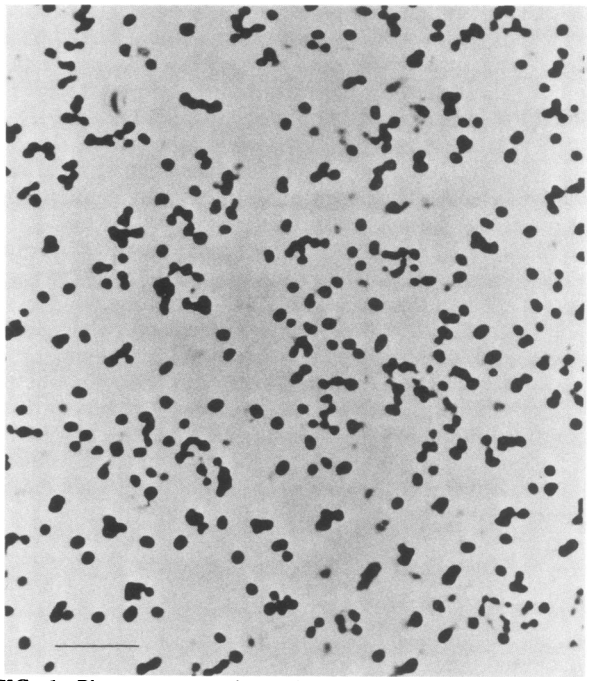

FIG. 1. Phase-contrast photomicrograph of strain SLP. The culture was at mid-exponential phase. Bar, 5 μm.

graphs. Cells were lysed easily in 0.001% Triton X-100 or 0.05% sodium dodecyl sulfate. The isolate also lysed in solutions of less than 0.3 M NaCl.

Growth and methane production were supported by methanol, methylamine, dimethylamine, and trimethylamine, with no growth occurring in H_2-CO_2, formate, acetate, methionine, choline, or betaine. No methane production or change in optical density occurred in these substrates after 60 days of incubation.

BES (5×10^{-3} M) inhibited growth and methanogenesis. This inhibition could not be reversed with additions of CoM at equal or greater concentrations. Antibiotic sensitivities were similar to the other *Archaeobacteria* (5, 6), with *d*-cycloserine and penicillin-G causing no decrease in growth or methanogenesis. Strain SLP was sensitive to cephalothin and tetracycline (all at 300 mg/liter).

For optimal growth, Na^+ and Mg^{2+} were required at concentrations higher than sea water. K^+, Ca^{2+}, and Fe^{2+} were needed at lower concentrations than those found in the south arm of the Great Salt Lake (14), but all were required for growth. KCl did not replace the NaCl requirement.

Maximum methane production occurred at an Na^+ concentration between 1.0 and 2.5 M, with the minimum generation times occurring at a concentration of approximately 2.0 M (Fig. 4). The optimal temperature for the isolate was 35°C, with little or no growth occurring at 45°C or greater (Fig. 5).

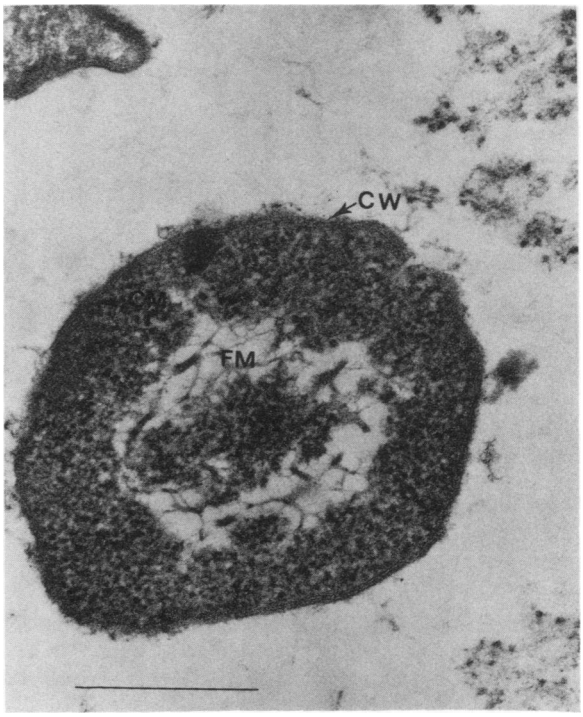

FIG. 3. Electron photomicrograph of strain SLP. Bar, 0.5 μm. CW, Cell wall; CM, cell membrane; and FM, fibrous material (possibly DNA).

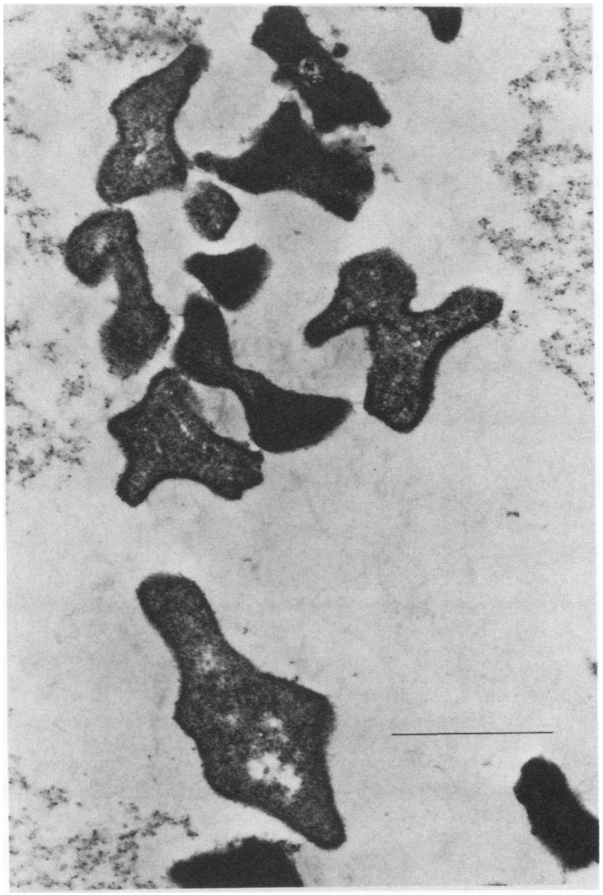

FIG. 2. Electron photomicrograph of strain SLP. The culture was at mid-exponential phase. Bar, 1 μm.

Maximum methane production occurred at a pH of 7.5 (Fig. 6). The DNA base ratio was 48.5 mol% G+C.

DISCUSSION

Strain SLP was found to be similar in some characteristics to a number of recently isolated methanogens. These include *Methanolobus tindarius* (8), *Methanococcoides methylutens* (19), *Methanococcus halophilus* (25), and strain SF1 isolated by Mathrani and Boone (10). The first three microorganisms were isolated from marine sediments, and the last was obtained from a solar saltern fed by sea water from San Francisco Bay. All the microorganisms, including strain SLP, are gram-negative, irregular cocci with thin cell walls sensitive to lysis by a number of agents and conditions. Methylamines and methanol are the only substrates utilized for growth and methanogenesis; thus, these five microorga-

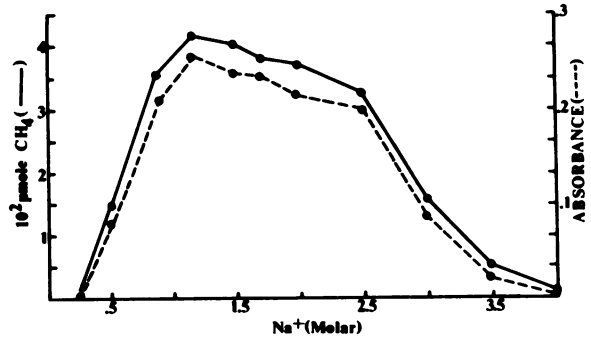

FIG. 4. Growth and methanogenesis of strain SLP at various sodium ion concentrations. Cultures were incubated at 37°C for 15 days.

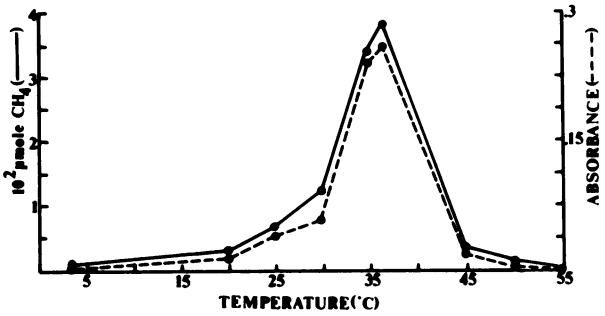

FIG. 5. Growth and methanogenesis of strain SLP incubated at various temperatures for 15 days.

nisms may represent a distinct group within the methanogenic bacteria.

Strain SLP differs from all the isolates except strain SF1 in salt requirement and temperature optimum. The sodium chloride requirements of the three marine isolates reflect their original habitats with a maximum required concentration reported as 1.2 M NaCl for *Methanococcus halophilus* (25). Strain SF1 (10) was reported to have a salt requirement similar to that of strain SLP (Fig. 4). These two isolates also were similar in temperature optimum (35 to 37°C), whereas the other three organisms had slightly lower profiles, with optimal growth ranges from 25 to 36°C.

There was no definite antigenic relationship between strain SLP and members of the families *Methanobacteriaceae*, *Methanomicrobiaceae*, and *Methanosarcinaceae* as revealed by indirect immunofluorescence serotyping (E. Conway de Macario, personal communication). However, Conway de Macario (personal communication) did find an extremely weak cross-reaction between strain SLP and a member of the *Methanococcaceae*. The recent isolates discussed above were not available at the time of the antigenic screening.

Due to the ecosystem from which strain SLP was isolated and the unique physical and physiological parameters as compared to other described genera of methanogens, we recommend that a new genus and species, *Halomethanococcus mahi*, be created. The other recently isolated methanogens with similar characteristics discussed above could be included in this genus. We believe that the creation of the genus, *Halomethanococcus* is fitting because of characteristically high optimum concentration range of sodium chloride reported for this microorganism and the others discussed. The validity of this classification could be tested by comparison of 16s rRNA sequences (1, 22). Strain SLP named *Halomethanococcus mahi* under this proposed schema would be a reasonable type species for this genus, as the organism is moderately halophilic and was isolated from Great Salt Lake, an ecosystem that is constantly hypersaline and has no direct connection to marine systems (17). We tender the species designation in honor of R. A. Mah for his noteworthy research history in the areas of anaerobic microbiology and methanogenic bacteria.

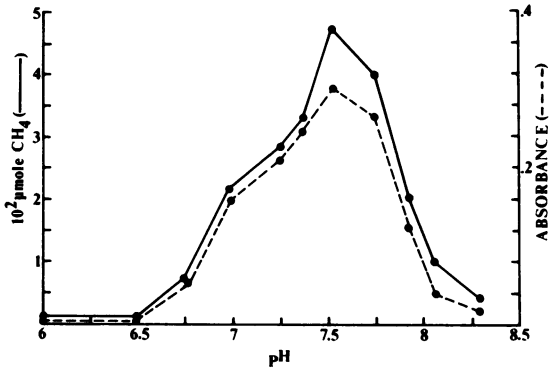

FIG. 6. Growth and methanogenesis of strain SLP incubated in media poised at various pH values. Cultures were incubated at 37°C for 15 days.

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