Isolation of an Aceticlastic Strain of *Methanosarcina siciliae* from Marine Canyon Sediments and Emendation of the Species Description for *Methanosarcina siciliae*

M. A. ELBERSON AND K. R. SOWERS*

Center of Marine Biotechnology, University of Maryland Biotechnology Institute,
Baltimore, Maryland 21202

A newly described strain of the genus *Methanosarcina* was isolated from submarine canyon sediments and is shown by comparative sequence analyses of 16S ribosomal DNA and the gene encoding methyl coenzyme M reductase, *mcrI*, to be a strain of *Methanosarcina siciliae*. Morphological and physiological characteristics are described. In contrast to the two previously described strains that grow exclusively on methanol, methylamines, and dimethylsulfide, *M. siciliae* C2J is also capable of growth on and methanogenesis from acetate. We propose that the species description for *M. siciliae* be amended to include aceticlastic strains.

Methanogenic biodegradation is a predominant process in anaerobic marine sediments that are subject to high organic loading, such as the elevated portions of marshes, nearshore sediments, and submarine canyons (5, 8, 9, 12, 13). In these environments anaerobic consortia of fermentative bacteria, acetogenic bacteria, and methanogenic archaea degrade polymers and monomers in a synergistic process that yields methane and carbon dioxide as the terminal products (21). Aceticlastic methanogens, such as *Methanosarcina* spp., have a pivotal role in marine anaerobic consortia since up to 70% of the methane produced from polymer degradation in sediments is derived from the methyl moiety of acetate (3, 13). However, only two aceticlastic methanogens, *Methanosarcina acetivorans* and *Methanosarcina frisia* (= *Methanosarcina mazei*), have been isolated from the marine environment (2, 7, 14). *Methanosarcina siciliae* was also isolated from the marine environment, but previously described strains do not utilize acetate for growth (10, 11). In this report we describe an aceticlastic methanogenic archaeon designated strain C2J that is phylogenetically similar to strains of *M. siciliae*. This strain has physiological characteristics that distinguish it from previously described strains, including the ability to utilize acetate for growth and methanogenesis.

Acetate-utilizing marine methanogenic archaea were enriched from a sediment core retrieved from the Sumner Branch of Scripps Canyon near La Jolla, Calif., at a depth of 19.8 m below the surface of the water. The sediments consisted of an interwoven mat of sand and partially degraded algae and sea grass. The sediment was black and had a strong sulfide odor. In addition, bubbles were observed rising from sediments when they were disturbed. Contents of the core were pooled under N₂. Sediment (10%, vol/vol) was transferred to sterile marine mineral medium that was prepared anaerobically under an N₂-CO₂ (4:1) atmosphere as described previously (14). Sodium acetate was included as the only catabolic substrate at a final concentration of 0.1 M. In the initial culture, there was a 6-day lag before methane production was observed, which then subsided after 55 days. In all subsequent transfers there was no lag in growth, and methane production subsided within

20 days. The predominant cells in enrichment medium were irregularly shaped cocci that occurred singly or in pairs. After four sequential transfers in enrichment medium, serial dilutions of the culture were transferred to agar-solidified medium in roll tubes (14). Several colonies of irregular cocci were isolated, and one colony, designated strain C2J, was selected for further study.

Colonies were characterized on solidified plating medium as described previously (15). Strain C2J grew as circular, convex, pale yellow colonies that averaged 0.5 mm and 2 mm in diameter after 21 days of incubation on medium that contained the catabolic substrates acetate and trimethylamine, respectively. During exponential growth in liquid culture cells were irregularly shaped cocci, 3.4 ± 0.5 μ m in diameter, and occurred singly or in pairs (Fig. 1). Motility was not observed. As cultures approached stationary growth, cells formed multicellular aggregates similar to those reported for *M. acetivorans* (14). Single cells and aggregates were lysed with 0.005% sodium dodecyl sulfate, which is a characteristic of protein S-layer cell walls reported for *Methanosarcina* spp. grown in marine media (15).

The 16S rRNA- and methyl coenzyme M reductase (*mcrI*)-encoding sequences of strain C2J were determined for phylogenetic analysis (17, 19). Cells harvested from a 100-ml culture were lysed with 1% (wt/vol) sodium dodecyl sulfate, and chromosomal DNA was isolated by ethanol precipitation (15). The gene encoding 16S rRNA was amplified by PCR with archaeon-specific forward deoxyoligonucleotide primer 0025e and universal reverse deoxyoligonucleotide primer 1525 (1). Reaction mixtures contained the following components in a final volume of 20 μ l in a 200- μ l dome top reaction tube: 1 $\times$ PCR buffer (Perkin-Elmer), 200 μ mol of each deoxyribonucleoside triphosphate, 15 mM MgCl₂, 0.025% (vol/vol) formamide, 20 pmol of each primer, 1 μ g of chromosomal DNA, and 0.25 U of AmpliTaq (Perkin-Elmer). PCR were controlled with a Peltier thermocycler (model PTC 200; MJ Research) programmed as follows: 95°C for 15 s, followed by 30 cycles consisting of 94°C for 15 s, 55°C for 30 s, and then 72°C for 5 min. The gene encoding *mcrI* was amplified by PCR as described previously (17). Plasmid libraries were generated by directly ligating PCR fragments into plasmid pCRII (Invitrogen) according to the manufacturer's recommendations. The plasmid libraries were screened for clones containing 16S ribosomal DNA (rDNA) or *mcrI* inserts by direct PCR of colonies. Briefly, a colony was transferred to the PCR mixture

* Corresponding author. Mailing address: Center of Marine Biotechnology, University of Maryland Biotechnology Institute, Columbus Center, Suite 236, 701 E. Pratt Street, Baltimore, MD 21202. Phone: (410) 234-8878. Fax: (410) 234-8899. E-mail: Sowers@umbi.umd.edu.

FIG. 1. Phase-contrast micrograph of strain C2J grown in trimethylamine. The image was made by using an Olympus Vanox model AHB-T microscope at a magnification of $\times 1,000$. Bar = 20 μm .

with a sterile toothpick. After the cells lysed during the initial heating cycle, the plasmid insert was amplified by using the primers and thermocycling conditions described above. For sequencing, plasmid DNAs from selected clones were purified with a Qiagen plasmid minikit and were amplified with an ABI Prism dye terminator cycle sequencing reaction kit (Perkin-Elmer) according to the manufacturer's recommendations. Both strands of 16S rDNA and *mcrI* were sequenced by using flanking and nested primers for 16S rDNA and flanking primers only for *mcrI*. The flanking primers included T7 and SP6, and the nested primers were universal for *Escherichia coli* 16S rRNA positions 519-536, 536-519, 907-926, 926-907, and 1406-1392 (6). Each reaction mixture was loaded onto an ABI model 373 automated sequencer, and the sequence was analyzed with ABI Prism, version 2.1.1.

The sequences were aligned with representative 16S rDNA and *mcrI* sequences in the GenBank database by using PILEUP, version 8 (Genetics Computer Group, Madison, Wis.). Based on a comparison of a 1,350-bp segment of rDNAs, strain C2J is phylogenetically related to *M. siciliae* and *M. acetivorans*, with similarities of 99.8 and 99.0%, respectively. Evolutionary distances expressed as estimated numbers of changes per 100 nucleotides were calculated from the percentages of similarity by using the correction of Jukes and Cantor (4). A dendrogram based on the unweighted pair group method with arithmetic averages indicates that strain C2J is phylogenetically most closely related to *M. siciliae* (Fig. 2). Since 16S rDNA sequences do not measure phylogenetic distances sufficient to define a species, the phylogeny of the isolate was confirmed by comparative sequence analysis of a 490-bp segment of *mcrI*, which measures smaller evolutionary distances (17). The isolate had 98.4 and 100% *mcrI* sequence similarity with *M. acetivorans* and *M. siciliae*, respectively, indicating that strain C2J is an *M. siciliae* strain.

Several morphological and physiological characteristics distinguish this strain from the previously described *M. siciliae*

strains, T4/M (= OCM 156 = DSM 3028) and HI350 (= OCM 210 = DSM 6564). Single-cell forms of C2J grown in medium containing trimethylamine are larger (diameter, $3.4 \pm 0.5 \mu\text{m}$) than strain T4/M cells (diameter, $2.5 \pm 0.2 \mu\text{m}$) grown in the same medium and larger than the strain HI350 cells (diameter, 1.5 to $3.0 \mu\text{m}$) described previously (11). Under similar growth conditions, strain C2J forms aggregates after achieving stationary growth, but strains T4/M and HI350 form aggregates during early exponential growth (10). In addition, strain C2J forms large multicellular aggregates up to 1 mm in diameter compared with strain T4/M, which forms smaller aggregates averaging 0.1 mm in diameter under the same growth conditions (data not shown).

The physiological characteristics of strain C2J were determined by measuring the increase in A_{550} (18-mm path length) with a Spectronic 21 spectrophotometer (Bausch and Lomb). The media used for physiological characterization contained the following constituents (per liter of demineralized water): NaCl, 23.38 g; Na_2CO_3 , 3.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 12.6 g; KCl, 0.76 g; Na_2HPO_4 , 0.6 g; NH_4Cl , 0.5 g; cysteine hydrochloride $\cdot \text{H}_2\text{O}$, 0.25 g; $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$, 0.25 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.14 g; and resazurin, 0.001 g. In addition, 1% (vol/vol) trace element solution was added (20). The final pH of the medium was adjusted to 6.8 unless indicated otherwise. Media were dispensed into anaerobe tubes (18 by 150 mm; Bellco Glass, Inc.), which were sealed under $\text{N}_2\text{-CO}_2$ (4:1) with a butyl rubber septum secured with an aluminum crimp seal. When we tested for hydrogen utilization, the tubes were purged with $\text{H}_2\text{-CO}_2$ (4:1) and pressurized to 70 kPa after inoculation. Media were sterilized at 121°C for 20 min. After autoclaving, methanol was added as the catabolic substrate to a final concentration of 0.1 M unless indicated otherwise.

Strain C2J is mesophilic, and the maximum rate of growth occurs at 35°C (Fig. 3A). This optimal temperature for growth is approximately 5°C below that reported for *M. siciliae* T4/M and HI350 (11). In addition, growth of strain C2J was observed at 20°C , which is 5°C below the lowest growth temperature reported for the other strains. No growth was observed at 45°C .

The optimal pH for growth was determined in medium prepared with organic buffers substituted for carbonate as described previously (14) (Fig. 3B). Strain C2J exhibited the maximum rate of growth at pH 6.0 to 7.0, a range which is similar to the optimal range reported for *M. siciliae* T4/M and HI350 (11).

FIG. 2. Comparative sequence analyses of 16S rDNAs from *M. siciliae* C2J and representative strains from GenBank. The dendrogram was constructed from evolutionary distance matrices with TREECON software by using parameters indicated in the text (18). The significance of each branch is indicated by a bootstrap value calculated for 100 subsets. Bar = 0.05 substitution/site.

FIG. 3. Effect of temperature (A), pH (B), and extracellular solute concentration (C) on the growth rate of strain C2J on methanol. Values are means $\pm$ 1 standard deviation for three to six replicate cultures.

The range of medium osmolalities that support maximum rates of growth were determined in medium that contained a range of NaCl concentrations as described previously (16) (Fig. 3C). Strain C2J has a maximum rate of growth at 0.6 to 1.3 osmol/kg (corresponding to addition of 0.2 to 0.6 M NaCl). Like other strains of *M. siciliae*, strain C2J grows poorly at 0.3 osmol/kg (no addition of NaCl) (11). Therefore, this newly described strain, like previously described strains of *M. siciliae*, is slightly halophilic, which distinguishes it from other species of the genus *Methanosarcina*, which are halotolerant (7, 16).

After at least three sequential transfers in medium containing either methanol, trimethylamine (0.1 M), or dimethylsulphide (4.4 mM) as a catabolic substrate, strain C2J exhibited specific growth rates of 0.14 ± 0.008 , 0.09 ± 0.002 , and $0.03 \pm$

0.005 h^{-1} , respectively, which are similar to growth rates reported for strain HI350 (11). However, unlike previously reported strains of *M. siciliae*, medium containing sodium acetate (0.1 M) as a catabolic substrate supported growth of strain C2J with a specific growth rate of $0.009 \pm 0.001 \text{ h}^{-1}$ after three sequential transfers (11). Acetate-grown strain C2J did not grow or produce methane when it was transferred to medium that contained sodium formate (0.1 M) or $\text{H}_2\text{-CO}_2$ as the sole substrate. Trypticase, yeast extract, Casamino Acids, and vitamins (each at a concentration of 0.1%, wt/vol) were not required for growth in minimal medium and did not significantly stimulate growth.

Our results show that strain C2J is a strain of *M. siciliae* based on comparative sequence analysis of 16S rDNA and *mcrI*. Characteristics that distinguish this strain from the previously described strains, T4/M and HI350, include larger cell size, formation of larger aggregates during stationary growth, and lower minimal and optimal growth temperatures. However, the most distinguishing trait is the ability of strain C2J to use acetate for growth and methanogenesis. The current description of *M. siciliae* as a nonacetate-utilizing, obligate methylotroph is hereby emended to include acetate-utilizing strains. The description is also emended to include a cell size range of 1.5 to 4 μm and a temperature range of 35 to 40°C for optimal growth. Strain C2J has been deposited in the Oregon Collection of Methanogens as OCM 653.

Nucleotide sequence accession number. The 16S rDNA nucleotide sequence of strain C2J has been deposited in the GenBank/EMBL Data Bank under accession no. U89773.

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