Isolation and Characterization of *Methanocorpusculum labreanum* sp. nov. from the LaBrea Tar Pits

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A new species of coccoid methanogen was isolated from the surface sediments of Tar Pit Lake at the LaBrea Tar Pits in Los Angeles, Calif. Surface colonies of strain Z^T (= OGC 1^T) (T = type strain) were tan, circular, clear, and convex with entire edges. The cells were irregular, nonmotile, and coccoid (diameter, 0.4 to 2.0 µm) and grew by producing methane from H₂-CO₂ or formate but not from methanol, methylamines, or acetate. They exhibited a negative Gram reaction, had protein cell walls, and were susceptible to lysis by sodium dodecyl sulfate. They grew over a narrow pH range (pH 6.5 to 7.5), with fastest growth near pH 7, and they were mesophilic (growth was most rapid at 37°C). Cells grew fastest with 15 g or less of NaCl per liter of medium. We propose that this isolate be named as a new species of methanogen, *Methanocorpusculum labreanum*, with strain Z (= Oregon Graduate Center Collection strain OGC 1 = DSM 4855) as the type strain.

Until recently, new species of coccoid H₂-utilizing methanogenic bacteria were assigned to one of two genera, *Methanococcus* or *Methanogenium*, based mainly on their guanine-plus-cytosine (G+C) contents (2, 9, 13, 15, 16). Recently, *Methanocorpusculum parvum* was described as a new genus and species; this coccoid organism might have been placed in the genus *Methanogenium* except that 16S ribosomal ribonucleic acid cataloging data indicated that it was significantly different from *Methanogenium cariaci*, the type species of that genus (18). Deoxyribonucleic acid (DNA)-DNA hybridization studies (L. Xun, D. R. Boone, and R. A. Mah, submitted for publication) showed great diversity among the species of *Methanogenium*, with levels of hybridization for *Methanogenium aggregans* of 32% or less with *Methanogenium marisnigri* JR1^T (T = type strain), *Methanogenium bourgense* MS2^T, *Methanogenium olen-tangyi* RC/ER^T, *Methanogenium thermophilicum* UCLA, and *Methanogenium tationis* DSM 2702^T. These DNA-DNA hybridization studies also showed that strain Z^T is closely related to *Methanogenium aggregans* and *Methanocorpusculum parvum*, but not so closely as to be placed in the same species. We report here the isolation and characterization of strain Z^T and propose that it should be named *Methanocorpusculum labreanum* sp. nov.

(Portions of the results were reported previously [L. Xun, D. R. Boone, and R. A. Mah, Abstr. Am. Soc. Microbiology 1988, 124, p. 185].)

MATERIALS AND METHODS

Sample collection. The sample was collected from an active, gas-producing area of Tar Pit Lake at the LaBrea Tar Pits in Los Angeles, Calif. A 250-ml polypropylene bottle was half-filled with surface (upper-5-cm) sediment, then filled to the top with water from the lake, and transported to our laboratory. The bottle was placed inside an anaerobic chamber and mixed to suspend the sediment, and 0.5 ml of the resulting slurry was inoculated into 5 ml of enrichment medium in a serum tube. This culture was removed from the chamber, and the gas phase was exchanged with H₂-CO₂.

Culture media. Enrichment medium contained (per liter of deionized water) 50 mM sodium formate, 2 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 2 g of yeast extract, 5 g of NaHCO₃, 1 g of NH₄Cl, 0.4 g of K₂HPO₄, 0.2 g of KH₂PO₄, 0.1 g of MgCl₂ · 6H₂O, 0.5 g of L-cysteine hydrochloride, and 0.2 g of Na₂S · 9H₂O. The gas phase was H₂-CO₂ (4:1) pressurized to 152 kPa; the final pH of the medium was 7.0.

Agar medium for roll tubes contained (per liter of deionized water) 15 g of sodium formate, 1.5 g of sodium acetate, 2 g of Trypticase peptone, 2 g of yeast extract, 1 g of NaHCO₃, 1 g of NH₄Cl, 0.4 g of K₂HPO₄, 0.2 g of KH₂PO₄, 0.1 g of MgCl₂ · 6H₂O, 0.8 g of NaCl, 0.5 g of L-cysteine hydrochloride, 0.2 g of Na₂S · 9H₂O, and 150 g of purified agar. The gas phase was N₂, and the pH was 6.8.

The liquid medium used for characterization of the isolate contained (per liter of deionized water) 2 g of Trypticase peptone, 2 g of yeast extract, 0.34 g of sodium acetate, 3.8 g of NaHCO₃, 2.5 g of NaCl, 1 g of NH₄Cl, 0.1 g of MgCl₂ · 6H₂O, 0.1 g of CaCl₂ · 2H₂O, 0.4 g of K₂HPO₄, 0.5 g of L-cysteine hydrochloride, 0.25 g of Na₂S · 9H₂O, 10 ml of a trace mineral solution (3), and 1 mg of resazurin. The medium was prepared with a gas phase of N₂-CO₂ (7:3), and the final pH was 7.2. After inoculation, pure H₂ (69 kPa of overpressure) was added.

Additions to the media were made from sterile, O₂-free stock solutions. These were normally sterilized by autoclaving, but antibiotic solutions were prepared fresh in O₂-free water and sterilized by filtration.

Culture methods. The modified (1) culture techniques of Hungate (4) were used throughout these studies. During growth on H₂-CO₂, gas was frequently replenished by adding H₂-CO₂ (3:1) to a total pressure of 151 kPa. Growth rates were determined from methane production, and the methane produced during growth of the inoculum was included when growth rates were calculated (11). During investigation of optimal pH, temperature, and salt concentration, methane production was not uncoupled from growth. This was established by noting an exponential increase in methanogenesis during growth and by transferring the late-exponential-phase cultures to fresh medium and observing the expected rates of methanogenesis. Experiments to determine optimal growth conditions were also performed with formate as the sub-

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strate in roll tube medium without agar; the growth responses to environmental conditions were similar although the absolute growth rates were slightly less.

Analytical methods. Methane was quantified by gas chromatography with thermal conductivity detection. DNA was obtained after lysing cells by adding 0.2 g of sodium dodecyl sulfate per liter; DNA was extracted by the method of Marmur (7), and its density was determined by centrifugation in CsCl gradients immobilized after centrifugation by polymerization of acrylamide (12). *Clostridium perfringens* (ρ was assumed to be 1.691 g/cm³ [14]; Sigma Chemical Co., St. Louis, Mo.) and *Micrococcus lysodeikticus* (ρ was assumed to be 1.731 g/cm³ [14]; Sigma) were used as internal standards for determining buoyant density. The G+C content was calculated from the density by using the formula of Schildkraut et al. (14). Epifluorescence was determined microscopically by using a type O2 filter set (Carl Zeiss, Inc., Thornwood, N.Y.); this provided an excitation spectrum with a peak at 365 nm and a cutoff at 395 nm and a 420-nm long-pass barrier filter.

RESULTS AND DISCUSSION

Isolation of strain Z^T. Enrichment medium with H₂-CO₂ as the substrate was inoculated with a sediment slurry from Tar Pit Lake. This enrichment culture was incubated at 37°C until methanogenesis was complete. The culture was serially diluted, and dilutions were inoculated into roll tube medium containing formate as the substrate. After the roll tube cultures were incubated for 1 week at 37°C, pinpoint colonies with epifluorescence characteristic of methanogens were observed. These colonies grew to a maximum diameter of 0.5 mm within 15 days after inoculation. An epifluorescent colony was picked, inoculated into liquid medium, and incubated until methanogenesis was complete. Microscopic examination revealed that the culture was not axenic, although it probably contained a single strain of methanogen. We maintained this mixed culture by periodic transfer into fresh medium. After several unsuccessful isolation attempts in which we diluted the culture and inoculated roll tube media, we grew the mixed culture in enrichment medium supplemented with (per liter of liquid culture medium) 0.1 g of D-cycloserine and 2 g of penicillin G, which eliminated most of the contaminating heterotrophs. We also included these antibiotics at the same concentrations in roll tube media which were inoculated with dilutions of this liquid culture. When these roll tube cultures were incubated, only pinpoint, epifluorescent colonies were observed; all surface colonies had a similar appearance, as did the subsurface colonies. A colony was picked, diluted in liquid medium without antibiotics, and inoculated into fresh roll tube medium without antibiotics. After incubation, the colonies which appeared were all epifluorescent. The surface colonies were tan, circular, clear, and convex with entire edges. A colony was picked and inoculated into liquid medium. This culture, strain Z^T, was axenic as indicated by microscopic examination and by its formation of a single colony type when it was inoculated into roll tube medium. It was subsequently deposited in the Oregon Graduate Center Collection of Methanogenic Archaeobacteria (Oregon Graduate Center, Beaverton) as strain OGC 1^T.

Morphology. The cells were coccoid and 0.4 to 2.0 μ m in diameter. The degree of irregularity was dependent on the physiological state of the cells and on the ionic strength of the medium; the cells were less irregular in liquid medium containing 5 g of NaCl per liter than in medium containing 20

FIG. 1. Effect of pH on the specific growth rate of strain Z^T.

or 25 g of NaCl per liter. In medium without added acetate growth was slower, and the cells were also more regular. Examination of cells in hanging-drop slides prepared in the absence of O₂ revealed no motility, and no flagella were observed in negatively stained electron micrographs. Thin sections were prepared by standard fixation techniques with glutaraldehyde and osmium and embedded in Spurr plastic. Transmission electron micrographs of these sections revealed a surface protein (diameter, about 25 nm) which was hexagonally closely packed.

Substrates. The ability of strain Z^T to grow on substrates other than H₂-CO₂ was tested by replacing H₂ with the following substrates (at concentrations of 20 mM): sodium formate, sodium acetate, sodium propionate, methanol, trimethylamine, and ethanol. Only sodium formate supported methanogenesis compared with controls without added substrate.

Optimal pH. The optimal pH for growth and methanogenesis was determined by inoculating strain Z^T into medium previously adjusted to various pH values by adding 1 M HCl or 1 M NaOH. Growth occurred over a narrow range of pH values (pH 6.5 to 7.5), and the optimum pH for growth was about 7.0 (Fig. 1).

Optimum temperature. Growth rates measured at various temperatures (Fig. 2) indicated an optimum temperature of about 37°C for this organism.

FIG. 2. Effect of temperature on the specific growth rate of strain Z^T.

FIG. 3. Effect of NaCl concentration on the specific growth rate of strain Z^T . The concentrations of Na^+ from other medium constituents were about 46 mM (equivalent to the Na^+ contribution of 2.6 g of NaCl per liter) for medium without acetate and 48 mM (equivalent to 2.8 g of NaCl per liter) for medium with 2.5 mM acetate.

Optimum NaCl concentration and effect of acetate on growth rate. Figure 3 shows that strain Z^T grew poorly in the presence of elevated levels of salt, both in the presence and in the absence of sodium acetate. At lower salt levels (less than 15 g of NaCl per liter), growth was more rapid and was stimulated by sodium acetate. At high salt concentrations acetate did not stimulate faster growth, perhaps because acetate is more stimulatory when conditions otherwise allow rapid growth.

Growth factor requirements. Strain Z^T required one or more organic compounds contained in Trypticase peptone or yeast extract (or cysteine). The organism was unable to grow when these compounds were omitted from the medium, even if acetate was included. Adding ash from Trypticase peptone and yeast extract did not relieve the requirement for the presence of these nutrients in the medium.

G+C content of the DNA. The G+C content of the DNA was 50 mol%.

Taxonomy. *Methanogenium*, *Methanococcus*, and *Methanocorpusculum* are the only genera which contain coccoid methanogens that are able to use $\text{H}_2\text{-CO}_2$ and formate as substrates. *Methanospiraera* (8) and some genera of the *Methanosarcinaceae* (5) contain coccoid members that are able to use $\text{H}_2\text{-CO}_2$, but members of these genera can also dissimilate methyl compounds, and none can use formate (6). The ability to use methyl compounds is an important taxonomic characteristic of methanogens (6). The DNA of strain Z^T had a G+C content of 50 mol%, indicating that it is not a member of the genus *Methanococcus*, whose members have a G+C content range of 31 to 33 mol%. Studies of interspecies hybridization of DNA (Xun et al., submitted) indicated that strain Z^T is more closely related to *Methanocorpusculum parvum* (31%) and *Methanogenium aggregans* (48%) than to other species of *Methanogenium* (–6 to 18%). The small coccoid morphology of strain Z^T is also similar to that of *Methanocorpusculum parvum* and *Methanogenium aggregans*. We propose *Methanocorpusculum labreanum* as a new species of methanogen, with strain Z as the type strain.

Description of *Methanocorpusculum labreanum* sp. nov. *Methanocorpusculum labreanum* (la.bre.an'um. N.L. neu. adj. *labreanum*, of the LaBrea Tar Pits, Los Angeles, Calif.) cells are irregular, nonmotile cocci that are 0.4 to 2.0 μm in

diameter and exhibit a negative Gram reaction. They have a protein cell wall and are susceptible to lysis by 0.02 g of sodium dodecyl sulfate per liter. Surface colonies are tan, circular, clear, and convex with entire edges. $\text{H}_2\text{-CO}_2$ and formate are the only substrates for growth and methanogenesis. Trypticase peptone or yeast extract is required, and acetate may be stimulatory.

Growth is most rapid at pH 7.0, in the presence of 0 to 15 g of NaCl per liter, and at 37°C.

The G+C content of the DNA is 50 mol%, as determined by its density in CsCl compared with the densities of *C. perfringens* and *Micrococcus lysodeikticus*.

The type strain is strain Z (= OGC 1 = DSM 4855), which was isolated from surface sediments of Tar Pit Lake at the La Brea Tar Pits in Los Angeles, Calif.

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