Isolation and Characterization of *Methanolobus bombayensis* sp. nov., a Methylophilic Methanogen That Requires High Concentrations of Divalent Cations

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Methanolobus bombayensis B-1^T (= OCM 438^T) (T = type strain) was isolated from Arabian Sea sediments obtained near Bombay, India. This strain grew on methylamines, methanol, and dimethyl sulfide, but it did not catabolize H₂-CO₂, acetate, or formate. The cells were nonmotile, irregular coccoids (diameter, 1.0 to 1.5 µm) and occurred singly. Electron micrographs revealed that a cell membrane and a protein cell wall were present. The cells grew fastest at mesophilic temperatures, at a neutral pH, and at salinity levels near the salinity level of the ocean, and they required about 30 mM divalent cations (Mg²⁺ and Ca²⁺). The cells grew in mineral medium, but growth was greatly stimulated by yeast extract and peptones. The guanine-plus-cytosine content of the DNA was 39.2 ± 0.1 mol%. A comparison of 16S rRNA sequences showed that strain B-1^T was phylogenetically related to *Methanolobus vulcani*, but the sequences of these organisms differed by 2%.

Some marine methanogens use either H₂-CO₂ (6–8, 11, 14, 34) or acetate (30), but many marine isolates and nearly all isolates obtained from hypersaline sources are obligately methylophilic (3, 17, 19, 26, 35, 36). Obligately methylophilic methanogens grow exclusively on methyl compounds (such as methyl amines and methanol). Some of these obligately methylophilic organisms can also catabolize dimethyl sulfide and methanethiol (7, 10, 22, 25). *Methanosphaera* species, which grow by using H₂ to reduce methanol to methane, are not considered obligate methylophilic, nor are *Methanosarcina* species that catabolize acetate or H₂-CO₂ as well as methylophilic substrates.

The family *Methanosarcinaceae* contains all known halophilic, methylophilic methanogens. These organisms include extreme halophiles (in the genus *Methanohalobium*), moderate halophiles (in the genus *Methanohalophilus*), and slight halophiles (in the genera *Methanobrevibacter* and *Methanococcoides* and one *Methanosarcina* species, *Methanosarcina siciliae*). *Methanosarcina siciliae* is a synonym of *Methanobrevibacter siciliae* (24).

In this paper we describe the isolation of a slightly halophilic, methylophilic methanogen that requires high concentrations of Mg²⁺, *Methanobrevibacter bombayensis* sp. nov. strain B-1^T (T = type strain).

MATERIALS AND METHODS

Source of inoculum. Strain B-1^T was isolated from an Arabian Sea sediment sample obtained near Bombay, India. The sample was transported to a laboratory under anoxic conditions within 24 h.

Media and culture techniques. Modifications (21) of the anaerobic techniques of Hungate (12) were used. The composition of SWM medium was (per liter) 1.0 g of Na₂SO₄, 30 g of NaCl, 0.7 g of KCl, 10 g of MgCl₂ · 6H₂O, 1.0 g of CaCl₂, 0.2 g of NaHCO₃, 0.1 g of K₂HPO₄, 0.25 g of NH₄Cl, 2 mg of

Fe(NH₄)₂(SO₄)₂, 2 g of yeast extract, 2 g of peptones, 1.0 mg of resazurin, and 10 ml of a trace element solution (33). All of the constituents except sulfide were dissolved in water, boiled, and cooled under an O₂-free mixture of N₂ and CO₂ (4:1). The medium was distributed to serum bottles under the same gas mixture, and then the bottles were sealed and autoclaved at 121°C for 20 min. Sulfide from a sterile anoxic stock solution was added before inoculation. The pH of the medium was 7.2. For solid medium, 18 g of purified agar per liter was added. For other experiments we used a modification of MSH medium (22), a bicarbonate-buffered medium containing yeast extract, peptones, and 29.2 g of NaCl per liter. This medium was modified by increasing the concentration of MgCl₂ · 6H₂O from 2.7 g to 6.1 g/liter. Methanol (20 mM) was added as a catabolic substrate. To measure the effect of pH on growth, the pH of the medium was modified by adjusting the ratio of N₂ to CO₂ in the gas. Adjusting the fraction of CO₂ resulted in media with pH values between 6.8 (100% CO₂) and 8.0 (100% N₂). Media with pH values lower than 6.8 were obtained from 100% CO₂ medium by adjusting the pH with HCl. Media with pH values higher than 8.0 were obtained from 100% N₂ medium by adding NaOH. The decreases in pH during growth (due to CO₂ production) were small; the greatest decreases in pH (0.25 pH unit) occurred during growth in medium that had an initial pH of 8.0.

Determination of catabolic substrates. The use of catabolic substrates was determined by inoculating cultures into media containing various catabolic substrates (soluble substrates at concentrations of 20 mM or gaseous substrates at partial pressures of 0.7 atm [ca. 71 kPa]) and monitoring methanogenesis. Methane formation was compared with the methane formation in controls lacking a catabolic substrate, and cultures producing significant amounts of methane were transferred to fresh medium. The methanogenic rates of these cultures were evaluated to determine whether methanogenesis was accompanied by growth. Those cultures that did not produce significantly more methane than the controls lacking substrate were checked for methanogenesis after at least 4 weeks of incubation.

Determination of growth rates. Specific growth rates were

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calculated from the amounts of methane produced during growth (5). The specific growth rate during exponential growth was analyzed by linear regression of the logarithm of the total amount of methane that accumulated (including the methane produced by the inoculum [27]) and time. Inocula were grown under conditions similar to the experimental conditions.

Analytical techniques. The amount of methane was determined by gas chromatography with flame ionization detection (17). DNA was isolated by the method of Marmur (18), and the DNA guanine-plus-cytosine content was determined by high-performance liquid chromatography after enzymatic hydrolysis (20). We examined cells microscopically with an epifluorescence microscope equipped with a type O2 filter set (Carl Zeiss, Inc., Thornwood, N.Y.).

The reverse transcriptase method was used to sequence the 16S rRNA. The samples used for electron microscopy were fixed in a cacodylate-buffered solution containing osmium tetroxide (10 g/liter) and glutaraldehyde (25 g/liter) (1); 30 mM Mg^{2+} was included in all of the buffers.

RESULTS AND DISCUSSION

Isolation. We inoculated sediment samples into four bottles containing SWM medium (2 ml of inoculum was inoculated into 18 ml of medium). Two bottles contained 50 mM acetate as the catabolic substrate, and two bottles contained 50 mM methanol. After 30 days of incubation at 35°C, the methanol cultures but not the acetate cultures had produced methane. One methanol enrichment culture was grown in medium containing vancomycin hydrochloride (0.1 g/liter) to decrease the proportion of nonmethanogenic contaminants, and this culture was inoculated into roll tube medium for further purification. We picked a well-isolated colony that appeared after 2 weeks of incubation and aseptically transferred it to liquid medium under a stream of O_2 -free N_2 . The resulting culture was diluted and reinoculated into roll tube medium. This procedure was repeated until a single type of colony was obtained. The resulting culture was determined to be axenic on the basis of a microscopic examination of wet mounts, the presence of a single colony type in roll tube medium, and the absence of growth in either SWM medium lacking substrate or nutrient broth supplemented with 10 g of glucose per liter. We designated this culture strain B-1^T and deposited it in the Oregon Collection of Methanogens as strain OCM 438^T.

Colonial and cell morphology. Surface colonies were circular, convex with entire margins, colorless, translucent, and 2 to 3 mm in diameter after incubation for 7 to 10 days. The cells were very irregular coccoids with diameters of 1.0 to 1.5 μm . The cells were nonmotile, and thin-section electron micrographs revealed no flagella. The Gram stain reaction was negative.

The cells lysed immediately when 0.1 g of sodium dodecyl sulfate per liter was added, indicating that a proteinaceous cell wall was present (6). Electron microscopy also revealed an S-layer (Fig. 1). When cells were suspended in a buffer having the same osmolality as their medium but containing no $MgCl_2$ or $CaCl_2$, they became turgid and spherical. In *Methanococcus voltaei*, Ca^{2+} and Mg^{2+} stabilize the cell envelope and are tightly bound to the wall protein (13); whether they serve the same function in strain B-1^T is unknown.

Methylotrophy and environmental factors for growth. Physiological studies conducted with modified MSH medium and with SWM medium gave similar results. Strain B-1^T catabolized methanol, trimethylamine, and dimethyl sulfide. Cultures containing H_2 and 20 mM methanol produced the same amount of methane as cultures containing only 20 mM meth-

FIG. 1. Thin-section electron micrograph showing a coccoid cell grown in liquid medium containing 30 mM Mg^{2+} .

anol. Cultures did not use formate, acetate, or H_2 - CO_2 . Cells grew fastest at neutral pH values (Fig. 2) and in the presence of about 0.5 M NaCl (Fig. 3). The levels of salinity tolerated by strain B-1^T, which grew in the presence of 2 M NaCl (Fig. 3), were higher than the levels of salinity tolerated by other *Methanobolus* strains (6). Strain B-1^T was mesophilic (Fig. 4). Using the square root model (15, 28), we analyzed the effect of temperature on the specific growth rate and found that strain B-1^T cells could not grow at 13.2°C or at temperatures below 13.2°C or at 45.7°C or at temperatures above 45.7°C and that

FIG. 2. Effect of initial pH on the specific growth rate of strain B-1^T. The results of three replicate measurements at each of five pH values are shown (some of the symbols overlap). The solid line connects the average growth rates at the pH values tested.

FIG. 3. Effect of NaCl on the specific growth rate of strain B-1^T. The results of three replicate measurements at each of eight levels of salinity are shown (some of the symbols overlap). The solid line connects the average growth rates at the levels of salinity tested.

they could grow fastest at 36.6°C (Fig. 4). However, we did not detect growth at 45°C.

Requirement for a high concentration of Mg²⁺. Strain B-1^T did not grow in MSH medium, which contained 13 mM Mg²⁺ and 3 mM Ca²⁺, unless the concentration of Mg²⁺ or Ca²⁺ was increased. When the Ca²⁺ concentration in the medium was 3 mM, cells grew well in the presence of 30 to 85 mM Mg²⁺, but they grew poorly or not at all in the presence of 20 mM Mg²⁺. Cells also grew in MSH medium when the concentration of Mg²⁺ was 13 mM if the Ca²⁺ concentration was increased to 20 mM. Although species belonging to the genera *Methanococcoides* and *Methanobolus* generally grow fastest in medium containing 40 to 60 mM Mg²⁺ (16, 17, 22, 31), they differed from strain B-1^T because they were able to grow in MSH medium containing 13 mM Mg²⁺ and 3 mM Ca²⁺ (data not shown).

Divalent cations play a significant role in biological systems and are known to maintain the ultrastructure of prokaryotes. Elevated Mg²⁺ and Ca²⁺ concentrations cause the disaggregation of *Methanosarcina* species and *Methanobolus taylorii*

FIG. 4. Effect of temperature on the specific growth rate of strain B-1^T. The results of three replicate measurements at each of six temperatures are shown (some of the symbols overlap). The solid line is the best fit (15) as determined by the square root model (28).

GS-16^T (= *Methanobolus* sp. strain GS-16 [4]) (2, 25). Also, these cations may increase the ability of sludge to settle during anaerobic waste treatment; the numbers of packets of coccoidal cells similar to *Methanosarcina thermophila* cells increase in the presence of Mg²⁺ in thermophilic upflow anaerobic sludge blanket reactors (29). *Methanospirillum* strains respond to medium supplemented with low concentration of Ca²⁺ by growing as long nonflagellated filaments (9).

Requirement for growth factors. Strain B-1^T grew in mineral medium (specific growth rate, 0.050 h⁻¹) when methanol was added as the catabolic substrate. However, it grew faster when other organic compounds were added; the compounds tested included 3 mM 2-mercaptoethanesulfonate (specific growth rate, 0.094 h⁻¹), 4 mM sodium acetate (specific growth rate, 0.106 h⁻¹), 5 mM glycine betaine (specific growth rate, 0.108 h⁻¹), a vitamin mixture (33) (specific growth rate, 0.107 h⁻¹), and 2 g of peptone per liter (specific growth rate, 0.129 h⁻¹). Fastest growth occurred when we added 2 g of yeast extract per liter and 2 g of peptones per liter (specific growth rate, 0.156 h⁻¹).

DNA analysis. The guanine-plus-cytosine content of the DNA was 39.2 ± 0.1 mol%. The 16S rRNA sequence of strain B-1^T was compared with the sequences of other methanogens and was found to be most similar to the sequences of *Methanobolus* strains, especially to the sequence of *Methanobolus vulcani*, from which it differed by 2%.

Taxonomy. The physiological and morphological characteristics and the results of 16S rRNA comparisons indicated that strain B-1^T belongs in the genus *Methanobolus*. In a previous classification, this genus contained three species, *Methanobolus tindarius*, *Methanobolus vulcani*, and *Methanobolus siciliae* (32). However, on the basis of the results of a 16S rRNA sequence comparison and phenotypic characterization tests, *Methanobolus siciliae* was transferred to the genus *Methanosarcina* as *Methanosarcina siciliae* (24). The results of previous studies also indicated that strain GS-16 (25) belongs in the genus *Methanobolus*, and the name *Methanobolus taylorii* has been proposed (4). *Methanohalophilus oregonensis* also belongs in the genus *Methanobolus*, and transfer of this species to the genus *Methanobolus* as *Methanobolus oregonensis* has been suggested but not formally proposed (6).

Comparisons of the strain B-1^T 16S rRNA sequence with the sequences of these and other methanogens indicated that strain B-1^T was most similar to *Methanobolus vulcani* PL-12/M^T, but that the sequences of these two organisms were 2% different. A difference of 2% in 16S rRNA sequences suggests that strains belong in separate species (6). Strain B-1^T differed from *Methanobolus vulcani* in some phenotypic characteristics. Unlike *Methanobolus vulcani* (23), strain B-1^T required a high concentration of divalent ions. Strain B-1^T was also slightly more alkaliphilic than *Methanobolus vulcani* PL-12/M^T; strain B-1^T grew well at pH 8 but did not grow at pH 6 (Fig. 2), whereas *Methanobolus vulcani* PL-12/M^T grew well at pH 6 but did not grow at pH 8 (unpublished data).

Strain B-1^T also differed phenotypically from other species that belong to the genus *Methanobolus*. Strain B-1^T differed from *Methanohalophilus oregonensis* WAL1^T (= "*Methanobolus oregonensis*") and *Methanobolus taylorii* GS-16^T in that it could not grow at pH values above 8. Strain B-1^T grew in mineral medium containing its catabolic substrate as the only organic addition (as does *Methanobolus tindarius* [16]), whereas *Methanohalophilus oregonensis* requires vitamins for growth (17). These phylogenetic and phenotypic distinctions indicate that strain B-1^T represents a new *Methanobolus* species. Thus, we propose a new species, *Methanobolus bombayensis*, with the description given below.

Methanobolus bombayensis sp. nov. *Methanobolus bombayensis* (bom.bay.en'sis. N. L. adj. *bombayensis*, from Bombay, indicating the source of the type strain). Cells are irregular, nonmotile coccoids (diameter, 1 to 1.5 μ m) and occur singly. Gram negative. Lysed by 0.1 g of sodium dodecyl sulfate per liter. Surface colonies are circular, convex, translucent, and colorless with entire margins and have diameters of 2 to 3 mm after 7 to 10 days of incubation.

The catabolic substrates used include methanol, trimethylamine, and dimethyl sulfide, but not formate, acetate, or H_2 - CO_2 . Grows in mineral medium containing a catabolic substrate; yeast extract and peptones are highly stimulatory.

Sodium and magnesium are required for growth. Fastest growth occurs in the presence of 0.5 M Na^+ and ≥ 0.03 M Mg^{2+} at pH 7.2 and 37°C.

The guanine-plus-cytosine content of the DNA is 39.2 ± 0.1 mol% (as determined by chromatographic analysis of bases).

The habitat is marine sediments. The type strain is strain B-1 (= OCM 438), which was isolated from Arabian Sea sediments obtained near Bombay, India.

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