Isolation and Characterization of a Dimethyl Sulfide-Degrading Methanogen, Methanolobus siciliae HI350, from an Oil Well, Characterization of M. siciliae T4/MT, and Emendation of M. siciliae

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We isolated strain HI350 from a gas and oil well in the Gulf of Mexico, characterized it, and found that it is closely related to Methanolobus siciliae T4/MT (T = type strain), which we also characterized. The previously published characterization of the type strain of M. siciliae was limited to the optimum temperature for growth, and our characterization suggested the species description given below. Cells are irregular, nonmotile, coccolid, and 1.5 to 3.0 μm in diameter. The catabolic substrates used include methanol, trimethylamine, and dimethyl sulfide, but not H₂CO₃, formate, or acetate. Growth is fastest in the presence of 0.4 to 0.6 M Na⁺, in the presence of 60 to 200 mM Mg²⁺, at pH 6.5 to 6.8, and at 40°C. Growth on trimethylamine is stimulated by yeast extract. An electrophoretic analysis confirmed that strain HI350 is closely related to strain T4/MT and indicated that major changes in the intracellular proteins of M. siciliae HI350 occur when the growth substrate is switched between dimethyl sulfide and trimethylamine.

Dimethyl sulfide is a major compound of the sulfur cycle in a wide variety of terrestrial and aquatic environments (1, 3, 6, 17). It has been estimated that this compound accounts for one-half of the biogenic input of volatile sulfur into the atmosphere, with nearly 75% of it being generated in marine regions (1). This dimethyl sulfide is thought to arise largely from the biodegradation of more complex sulfur compounds, such as dimethyl sulfoniopropionate, an osmoregulatory compound that is found in certain species of algae and higher plants (8, 20, 25). The emission of dimethyl sulfide has been linked to acid rain and even to the regulation of global climate (3, 20). However, microbial metabolism of dimethyl sulfide may decrease its outward flux.

Dimethyl sulfide is consumed by both sulfate-reducing and methanogenic bacteria in various anoxic sediments (6, 7, 20, 23, 24). When this compound is present at low concentrations (<10 μM), it is used mainly by sulfate reducers, but at higher concentrations dimethyl sulfide is used as a noncompetitive substrate for methanogenesis (7). Dimethyl sulfide supports growth and methanogenesis by the following obligately methylotrophic methanogens: strain GS-16 (14), Methanohalophilus zilhiae WeN5T (T = type strain) (12), and Methanohalophilus oregonensis WAL1T (9).

In this paper we report the isolation of a slightly halophilic methanogen, strain HI350, from an oil well at High Island in the Gulf of Mexico. This strain is very similar to Methanobacterium siciliae T4/MT (19). However, the previously published characterization of strain T4/MT was limited to its response to temperature (temperature range, 20 to 48°C; optimum temperature, 37°C) and G+C content (41.5 mol%) (19), so we characterized both strain T4/MT and strain HI350. Both of these organisms were able to use dimethyl sulfide (as well as trimethylamine and methanol) for growth and methanogenesis.

MATERIALS AND METHODS

Source of inoculum and bacterial strains. Water samples were collected from an offshore production platform receiving liquid and gas from oil well HI350 at High Island in the Gulf of Mexico. Aqueous, anoxic samples were transported to the laboratory within 48 h. The following strains were obtained from the Oregon Collection of Methanogens (OCM): Methanolobus siciliae T4/MT (= OCM 156T), Methanolobus tindarius Tindari T3T (= OCM 150T), Methanolobus vulcani PL-12/MT (= OCM 157T), Methanococcoides methylutens TMA-10T (= OCM 158T), and strain GS-16 (= OCM 58).

Media and culture techniques. The anaerobic techniques of Hungate (5) were used. The MSHCO₂ medium used throughout this study contained (per liter) 2.0 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 2.0 g of yeast extract, 0.5 g of 2-mercaptoethanesulfonic acid, 29.2 g of NaCl, 4.0 g of NaOH, 2.7 g of MgCl₂·6H₂O, 0.45 g of KCl, 1.0 g of NH₄Cl, 0.4 g of K₂HPO₄, 3H₂O, 0.25 g of Na₂S·9H₂O, 1.0 mg of resazurin, 5.0 mg of Na-EDTA·2H₂O, 1.5 mg of CoCl₂·6H₂O, 1.0 mg of MnCl₂·4H₂O, 1.0 mg of FeSO₄·7H₂O, 1.0 mg of ZnCl₂, 0.4 mg of AlCl₃·6H₂O, 0.3 mg of Na₂WO₄·2H₂O, 0.2 mg of CuCl₂·2H₂O, 0.2 mg of NiSO₄·6H₂O, 0.1 mg of H₂SeO₃,

FIG. 1. Phase-contrast micrograph of strain HI350.
FIG. 2. Production of methane and sulfide from dimethyl sulfide by strain HI350. Liquid dimethyl sulfide (2 mmol) was added to a 1-liter stainless steel culture bottle containing 500 ml of MSHCO2 medium. The amount of soluble H2S plus HS− was measured (●) and used to calculate the total sulfide concentration (including gaseous) (▲). The uninoculated culture containing dimethyl sulfide exhibited no increase in sulfide concentration, and the culture without dimethyl sulfide did not produce methane.

0.1 mg of H3BO3, and 0.1 mg of NaMoO4 · 2H2O. The medium was prepared by dissolving the NaOH in O2-free water and equilibrating the preparation with 100% CO2. The other constituents (except sulfide) were then added from concentrated stock solutions. The medium was dispensed into serum tubes and bottles which were sealed and autoclaved (121°C, 20 min). The pH of the medium was 6.8 after cooling and gas reequilibration. The enrichment medium used was the same as MSHCO2 medium but without mercaptoethanesulfonate and with the concentrations of Trypticase peptone and yeast extract reduced to 0.5 g/liter. For solid medium, 18 g of purified agar per liter was added. Media of various salinities were prepared by adding sodium or magnesium as their chloride salts. Media having pH values of 8.2 and 7.2 were prepared by replacing the headspace gas in the culture vessels with N2 and N2-CO2 (7:3), respectively. Other pH values were obtained by adding sterile, anoxic 1 M NaOH or HCl to one of these media. Sulfide and most substrates were added from sterile anoxic stock solutions approximately 1 h prior to inoculation. Methanethiol and dimethyl sulfide were sometimes added as gases. Cultures were tested in triplicate and were incubated at 40°C, and experiments were repeated at least twice. The experiments in which dimethyl sulfide was used as the

FIG. 3. Analysis of whole-cell proteins by denaturing gel electrophoresis of strain HI350, strain T4/M7, and related methanogens grown on trimethylamine (or dimethyl sulfide [DMS] where indicated). The diagram at the bottom indicates the positions and relative darkness of protein bands as determined visually by observing the gel on a light box. Mcoi., Methanococcoides; Mlob., Methanolobus.
substrate were carried out by using 1-liter portions of medium in 2-liter vessels fitted with butyl rubber stoppers and serum closures, except that the experiment in which we examined fermentation balance for dimethyl sulfide degradation was carried out in a 1-liter stainless-steel can equipped with a Teflon valve (to prevent loss of dimethyl sulfide or methanethiol into stoppers); for these experiments there were no replicates, but the experiments were repeated three times with similar results.

**Determination of optimal growth conditions.** Specific growth rates were determined by measuring the amount of methane formed during growth (2). The specific growth rate during exponential growth was analyzed by linear regression of the logarithm of total methane accumulated (including inoculum-produced methane [16]) and time. When the effects of environmental parameters (pH, temperature, salt concentrations) were tested, growth rates were determined by using cultures adapted to those conditions. We transferred cultures under those conditions at least two or three times sequentially, and we continued making sequential transfers until the specific growth rate was stable. In particular, shifts to a medium of lower osmolarity sometimes caused cells to lyse, even when adapted cells later grew well in that medium. To obtain cultures at a lower osmolarity, it was sometimes necessary to transfer a culture in several steps to media having progressively lower osmolalities.

The pH increased slightly during growth (about 0.1 pH unit) in medium at pH 6.8. Cultures at some other pH values had greater shifts in pH (as much as 0.4 pH unit), but in those cultures we determined the specific growth rate during the first one-half of the exponential phase, when the pH had changed by 0.2 pH unit or less.

**Analytical techniques.** Methane production was determined by gas chromatography with flame ionization detection (10). Whole-cell proteins were analyzed by denaturing slab gel electrophoresis, using 35 to 40 μg of protein per lane (9). The proteins were prepared from exponential-phase cells by extraction with detergent buffer (9). Sulfide was assayed by using the methylene blue method (21). We isolated DNA by using the method of Marmur (11), and W. B. Whitman (University of Georgia, Athens) determined DNA G+C contents by high-performance liquid chromatography after enzymatic hydrolysis (13). Microscopy for colony observation and purity checks was done with an epifluorescence microscope equipped with a type O2 filter set (Carl Zeiss, Inc., Thornwood, N.Y.).

**RESULTS AND DISCUSSION**

**Isolation.** The aqueous portion of a down-hole oil well sample was diluted serially in enrichment medium, and 0.1 ml of each dilution was inoculated into tubes containing MSHCO2 enrichment medium supplemented with 20 mM trimethylamine and pressurized with a partial pressure of H2 of 101 kDa. Methanogenesis occurred only in low-dilution tubes (>10 μl of the original sample). After three weeks of incubation, the methane-producing culture of the highest dilution was transferred to fresh media containing a single substrate, either trimethylamine or H2-CO2. Subsequent transfers showed that methane was readily produced from either of the substrates, but only the trimethylamine-using culture was used to inoculate agar roll tubes for isolation in this study. Well-isolated colonies with strong autofluorescence appeared after 10 days, and one of these colonies was picked, diluted, inoculated into a second set of roll tube media for further purification, and incubated. This procedure was repeated until a single colony type was observed and then repeated two additional times to assure purity. One colony was then transferred to liquid MSHCO2 medium and named strain HI350. No contaminants were detected during the growth of this culture, and no growth was observed when this culture was inoculated into MSHCO2 medium without substrates or into fluid thioglycolate medium. Strain HI350 was deposited in the Oregon Collection of Methanogens as strain OCM 210.

**Morphology.** Surface colonies of strain HI350 were circular, convex with entire margins, and dark yellow, and reached a diameter of 2.0 mm in 2 weeks. The cells were irregular, coccolid with a diameter of 1.5 to 3.0 μm (Fig. 1), and stained gram negative. During the early exponential phase, the cells occurred singly or in pairs, but tended to form large clumps during the mid- to late-logarithmic growth phase. Motility was not observed. Both individual cells and cell clumps lysed immediately when sodium dodecyl sulfate (1 g/liter) was added. Cells of strain T4/M1 were similar in size, but cell clumps were not observed during any growth stage.

**Catabolic substrates.** Both strain HI350 and strain T4/M1 used methanol, trimethylamine, and dimethyl sulfide for growth and methanogenesis, but they did not use formate, H2-CO3, or acetate. Each strain produced methane from dimethyl sulfide; the experiments described below showed that strain HI350 can catabolize dimethyl sulfide. Strain
DIMETHYL SULFIDE-DEGRADING METHANOLOBUS SICILIAE

A. T4/M

B. HI350

C

Mg$^{2+}$ (M) vs Na$^+$ (M) diagram with various concentrations marked.

Sea water

0.29
H1350 was grown in medium containing 5 mM trimethylamine plus 5 mM dimethyl sulfide and in medium containing 5 mM trimethylamine alone. Within 2 to 3 days, each of these cultures produced the quantity of methane stoichiometrically expected by trimethylamine degradation, and methane production stopped. After about 10 days, the culture containing dimethyl sulfide began producing methane again, and this continued until the quantity of methane predicted from dimethyl sulfide degradation was formed. (\textit{Methanolobus sículæ} T4/M\textsuperscript{T} behaved similarly.) Initially, cultures of strain H1350 or T4/M\textsuperscript{T} growing on dimethyl sulfide had lags of 2 to 3 weeks before methanogenesis began, even when inocula were grown on dimethyl sulfide. When cultures of strain H1350 were transferred before methanogenesis was complete, the lag was shorter, and after several transfers with dimethyl sulfide as the catabolic substrate, the growth rate increased and the lag disappeared. When dimethyl sulfide-grown cultures were switched back to trimethylamine, growth began without a lag.

Strain H1350 formed approximately the stoichiometrically expected quantities of both sulfide and methane during growth on dimethyl sulfide (Fig. 2) according to the following equation:

\[(\text{CH}_3)_2\text{S} + \text{H}_2\text{O} \rightarrow \frac{1}{2}\text{CO}_2 + \frac{1}{2}\text{CH}_4 + \text{H}_2\text{S} \]  
\((1)\)

The formation of approximately 1.5 times as much methane as sulfide also indicated that there was complete demethylation of dimethyl sulfide. Cultures were transferred 12 times with dimethyl sulfide as the catabolic substrate. When 1 mM methanethiol was added as the sole catabolic substrate to cultures grown on dimethyl sulfide, methanogenesis proceeded without an extended lag, which also supported the possibility that methanethiol was an intermediate in dimethyl sulfide degradation:

\[(\text{CH}_3)_2\text{SH} + \frac{1}{2}\text{H}_2\text{O} \rightarrow \text{CH}_3\text{SH} + \frac{1}{2}\text{CO}_2 + \frac{1}{2}\text{CH}_4 \]  
\((2)\)

\[\text{CH}_3\text{SH} + \frac{1}{2}\text{H}_2\text{O} \rightarrow \text{H}_2\text{S} + \frac{1}{2}\text{CO}_2 + \frac{1}{2}\text{CH}_4 \]  
\((3)\)

The sum of equations 2 and 3 is equation 1 above. Another possibility is that dimethyl sulfide and sulfide are first converted to two methanethiol molecules and that methanethiol is the precursor for methanogenesis:

\[(\text{CH}_3)_2\text{S} + \text{H}_2\text{S} \rightarrow 2\text{CH}_3\text{SH} \]  
\((4)\)

\[2\text{CH}_3\text{SH} + \text{H}_2\text{O} \rightarrow 2\text{H}_2\text{S} + \frac{1}{2}\text{CO}_2 + \frac{1}{2}\text{CH}_4 \]  
\((5)\)

These reactions would account for the small delay in the appearance of \(\text{H}_2\text{S}\) compared with \(\text{CH}_4\) (Fig. 2), although this mechanism is not suggested by other data and there are other possible explanations for the delay in the appearance of sulfide.

The whole-cell proteins as analyzed by electrophoresis (Fig. 3) indicated that substantial changes in intracellular proteins occurred when cultures were shifted between trimethylamine and dimethyl sulfide as catabolic substrates. Strain H1350 grew more rapidly on methanol (0.13 ± 0.0025 h\textsuperscript{-1}) than on trimethylamine (0.08 ± 0.002 h\textsuperscript{-1}), whereas strain T4/M\textsuperscript{T} grew at similar specific growth rates (0.10 ± 0.004 h\textsuperscript{-1}) on both substrates. After adaptation of strain H1350 to growth on dimethyl sulfide, the growth rate was about 0.032 to 0.035 h\textsuperscript{-1}.

\textbf{Physiology.} Each strain exhibited good growth at pH values between 5.8 and 7.2 and grew most rapidly at pH 6.5 to 6.8 (Fig. 4). Figure 5 indicates that each strain required about 0.4 to 0.6 M Na\textsuperscript{+} and 60 to 200 mM Mg\textsuperscript{2+} for fastest growth. Each strain required at least small quantities of both of these cations, with no growth occurring whenever one was absent. Figure 5 shows that the growth rates were high only when both Na\textsuperscript{+} and Mg\textsuperscript{2+} were present. These cations are not only obligately required, but they also function together to moderate the water activity of the medium. Rapid growth required an Na\textsuperscript{+} concentration of ≥80 mM and an Mg\textsuperscript{2+} concentration of ≥20 mM, with the total of the Na\textsuperscript{+} and Mg\textsuperscript{2+} concentrations sufficient to provide a water activity between 0.96 and 0.985. When one of the cations was present in small quantities, the other had to be present in higher concentrations to provide the optimal water activity of the culture medium. The effects of Na\textsuperscript{+} and Mg\textsuperscript{2+} on the growth rates of the nonmethanogenic halophiles \textit{Halobacterium salinarium}, \textit{Halobacterium halobium}, and \textit{Halobacterium volcanii} have been attributed to other effects in addition to water activity (4), but the proportions of these two cations (whenever the concentration of each was above some minimum level and the water activity was optimal) had little effect on the specific growth rate of \textit{Methanolobus sículæ}.

Each strain was mesophilic, with fastest growth occurring at 40°C. No growth was observed at or above 50°C or at or below 20°C (Fig. 6). Previous determinations (19) indicated that the optimum temperature was 37°C and the maximum temperature for growth was 48°C. This maximum is consistent with our data for \textit{Methanolobus sículæ} T4/M\textsuperscript{T} (growth at 45°C but not at 50°C), but not with our data for strain H1350 (which was unable to grow at a temperature of 45°C or above). This is especially important because the maximum growth temperature was given as the major differentiating characteristic of \textit{Methanolobus} species (19). Our findings suggest that there is variation in the maximum temperature among \textit{Methanolobus sículæ} strains and that this characteristic should not be relied on to differentiate \textit{Methanolobus sículæ} from other \textit{Methanolobus} species or \textit{Methanococoides} species. Both strain T4/M\textsuperscript{T} and strain H1350 were able to grow in mineral medium supplemented with a single organic catabolic substrate, but yeast extract stimulated growth (Fig. 7).

\textbf{DNA composition.} The DNA of strain H1350 had a G+C content of 42.80 ± 0.08 mol\% and contained no significant modified adenine (≤0.14 ± 0.06 mol\%). Strain T4/M\textsuperscript{T} was reported to have a G+C content of 41.5 mol\% (19), but the method used to determine this value was not reported.

\textbf{Taxonomy.} The genera \textit{Methanococcoides} (18), \textit{Methanolobus} (19), \textit{Methanohaloophilus} (15), and \textit{Methanohalo- bium} (22) are the four currently recognized genera which include halophilic, obligately methylotrophic, cocoid meth-
anogens. Strain HI350 is only slightly halophilic, so it does not belong in the extremely halophilic genus Methanohalophilus. The genus Methanohalophilus contains three moderately halophilic species; two of these species are alkaliphilic (9, 12), and the other, Methanohalophilus mahii SLPL (15), requires much higher salinity (1.2 M Na+) than strain HI350 does. Thus, strain HI350 does not belong in this genus. Strain HI350 also differs from unassigned dimethyl sulfide-degrading species (15), which is slightly alkaliphilic. Comparisons of proteins (Fig. 3) also showed that there are significant differences between strains GS-16 and HI350.

Although members of the genera Methanococcoides and Methanolobus have somewhat similar morphological and physiological characteristics, our electrophoretic comparison of whole-cell proteins (Fig. 3) demonstrated that strain HI350 has protein band patterns that are very similar to those of Methanolobus siciliae T4/M\(^T\) and significantly different from those of Methanohalophilus vulcani PL-M\(^2\), Methanobacterium tindarius Tindari 3\(^T\), and Methanococcoides methylyfutens TMA-10\(^T\). This suggests that strain HI350 is a strain of Methanolobus siciliae. This assignment is also supported by similarities between strain T4/M\(^T\) and strain HI350 in cell size, absence of flagella, substrate range, optimal growth conditions, and nutritional requirements.

Thus, we propose the emended description of Methanolobus siciliae given below.

**Emended description of Methanolobus siciliae.** Methanolobus siciliae Stetter and König 1989 (si.ci.'li.ae. L. n. gen. siciliae, from Sicily, the isolation source of the type strain) cells are irregular, nonmotile, and coccoid (diameter, 1.5 to 2.0 μm), occur singly or sometimes in aggregates, and stain Gram negative. They are lysed by 1 g of sodium dodecyl sulfate per liter.

Surface colonies are dark yellow, circular, and convex with entire edges and attain a diameter of 2.0 mm in 2 weeks.

Trimethylamine, methanol, dimethyl sulfide, and perhaps methanethiol are catabolic substrates, but H\(_2\)-CO\(_2\), formate, and acetate are not. No organic compounds other than a catabolic substrate are required for growth, but yeast extract stimulates growth.

Sodium and magnesium are required for growth. Growth is most rapid in the presence of 0.4 to 0.6 M Na\(^{+}\), in the presence of 60 to 200 mM Mg\(^{2+}\), at pH 6.5 to 6.8, and at 40°C.

The G+C content of the DNA is 42 to 43 mol%.

The known habitats are anoxic marine sediments and saline subsurface sediments.

Strain T4/M (= OCM 156 = DSM 3028), the type strain, was isolated from marine sediment, and strain HI350 (= OCM 58) was isolated from an oil well.


