**Methanohalophilus oregonense** sp. nov., a Methylotrophic Methanogen from an Alkaline, Saline Aquifer

YITAI LIU, DAVID R. BOONE,* AND CHEE CHOI

Oregon Graduate Institute of Science and Technology, Beaverton, Oregon 97006-1999

*Methanohalophilus oregonense* WAL1T (= OGI 99T = DSM 5435T) (T = type strain) was isolated from an anoxic aquifer (pH 10, with 100 g of dissolved solids per liter of pore water) 3 m deep near Alkali Lake, an alkaline, desert lake in south central Oregon. An examination of the subsurface sediments revealed no methanogens that were capable of growth on methanogenic substrates, such as H4-CO3, formate, or acetate, which is consistent with the results of other studies of hypersaline, sulfate-containing anoxic environments. Strain WAL1T grew on trimethylamine and grew slowly on methanol or dimethylsulfide, but did not catabolize H4-CO3, formate, or acetate. The cells were irregular coccois (diameter, 1 to 1.5 μm), and cells growing in liquid media also formed clumps of 2 to 15 or more cells. The cells were mesophilic and required one or more vitamins present in yeast extract. Like the only previously described strain of alkaliophilic, methylotrophic methanogen (*Methanohalophilus zhilinae* WeNST), strain WAL1T grew most rapidly in medium of moderate salinity; strain WAL1T grew well in the presence of 0.1 to 1.4 M Na+ and grew most rapidly at an Na+ concentration of 0.35 M (specific growth rate, 0.1 h). Best growth occurred with about 50 mM Mg2+ and at a pH of 8.4 to 9.0. K+ appeared to be required, with 13 to 130 mM K+ supporting most rapid growth. The guanine-plus-cytosine content of the DNA was 40.9 ± 0.1 mol%.

Catabolic processes in anoxic environments are profoundly affected by the presence of sulfate. When sulfate is present, methanogenesis is inhibited because sulfate is preferred to CO2 as a terminal electron acceptor (1, 19), although methylamines continue to be used by methanogens (13). The catabolism of methylamines was first reported in mixed, methane-forming cultures (21) and appears to be universal among the members of the *Methanosarcinaeae*; the only genus outside that family which catabolizes methyl compounds to methane is *Methanosphaera* (5). Sulfate is often present in hypersaline environments, and under such conditions methylamines and perhaps other methylated alcohols, such as dimethylsulfide, are the major methanogenic substrates; methanogenesis from acetate, formate, or H2 is not significant. In this paper we report the isolation of a new, methylotrophic methanogen from such an environment. *Methanohalophilus oregonense* sp. nov. strain WAL1T (T = type strain) was isolated from anoxic subsurface sediments near West Alkali Lake in Oregon.

(Portions of the data were presented on a poster at the 1989 Northwest Branch Meeting of the American Society for Microbiology, Seattle, Wash., and at the 1988 Federation of European Microbiological Societies Symposium on the Microbiology of Extreme Environments, Tróia, Portugal.)

**MATERIALS AND METHODS**

**Site description and sample collection.** Alkali Lake and West Alkali Lake are hypersaline, alkaline desert lakes in the high desert of south central Oregon. These two lakes were a single lake in former times, but today they are separated by a narrow, shallow valley and a line of artesian springs. The water table in this valley is within about 1 m of the ground surface and flows away from the line of springs to each lake (14). The pH of the groundwater is about 10, with about 100 g of dissolved solids per liter (14).

We hand-argued a well that was 100 mm in diameter and approximately 2.8 m deep and was located 15 m southwest of the southwest shore of West Alkali Lake. The ground surface in this area was saline, and ground cover was sparse. We installed a polyvinyl chloride casing without a screen and open at the bottom. After we emptied the well by using a propane-powered pump, the water level recovered in 15 min to within approximately 1 m of the ground surface. Then we used the pump to recirculate the water within the well until a high-salinity slurry (containing solids from 2.8 m deep) developed. The recirculating stream had a temperature of 18°C. A part of the recirculating stream was diverted to a serum bottle, which was filled and allowed to overflow. We continued pumping the sediment slurry into the overflowing bottle until settling solids accumulated to a level approximately half-filling the bottle. The bottle was sealed with a butyl rubber stopper. A syringe needle was placed through the stopper during stopper insertion to allow displaced liquid to escape as the stopper was pushed into place; thus, the bottle was sealed without a gas headspace. Samples of the water from this well were also collected in a separate container.

**Media and culture techniques.** Anaerobic techniques of Hungate (8) were used. We used MSHA medium, which was similar to MS medium (4) and contained (per liter) 20 mmol of trimethylamine hydrochloride, 2.0 g of yeast extract, 2.0 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 0.5 g of 2-mercaptoethanesulfonic acid, 39 g of NaCl, 4.0 g of NaOH, 1.7 g of MgCl2 · 6H2O, 1.5 g of KCl, 1.0 g of NH4Cl, 0.4 g of K2HPO4, 3H2O, 0.25 g of Na4S · 9H2O, 1.0 mg of resazurin, 5.0 mg of Na2-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, 0.1 mg of H4BO3, and 0.1 mg of Na2MoO4 · 2H2O. The medium was prepared by dissolving NaOH in O2-free water and equilibrating the preparation with 100% CO2. The other constituents (except sulfide) were added, and the medium was distributed to serum tubes, which were sealed and autoclaved (121°C, 20 min). Precipitates formed during autoclaving redissolved when the medium was cooled and

* Corresponding author.
equilibrated with CO₂ gas. Before use, the gas phase was exchanged with 100% N₂, sulfide was added, and the pH was adjusted to 8.5 with NaOH. For solid media, we added 18 g of purified agar per liter to the medium and maintained it in suspension by stirring with a magnetic stirrer as we distributed the medium to tubes.

Enrichment medium was the same as the medium described above, except that it contained 0.5 g of Trypticase peptone per liter and 0.5 g of yeast extract per liter, KCl was omitted, and it was made up with 10% (vol/vol) filter-sterilized water from the site.

 Cultures were grown statically at 37°C in MSHA medium containing 20 mM trimethylamine as the catabolic substrate. Inoculum volumes were 5% of culture volumes. Determinations of optimal conditions were carried out under the same conditions except as indicated below.

**Determination of optimal conditions.** Optimal conditions for growth were determined by measuring specific growth rates under various conditions. We quantified growth by measuring methane formation. The cells present at inoculation were accounted for by the methane formed during growth. When a culture grew more slowly than it did under optimal conditions, we used such a culture as inoculum for a second determination of growth under the same conditions and continued this procedure if necessary to be sure that the culture was adapted to these conditions and that methanogenesis was coupled to growth.

To determine the optimal pH for growth, we varied the pH of the medium by adding sterile, anoxic 1 M HCl and 1 M NaOH solutions. The pH values reported below are the initial pH values, but the pH values varied less than 0.2 pH unit during growth of the inoculum culture (17). The specific growth rate during exponential growth was analyzed by linear regression of the logarithm of total methane (including inoculum-produced methane) accumulated and time. When a culture grew significantly more slowly than it did under optimal conditions, we used such a culture as inoculum for a second determination of growth under the same conditions and continued this procedure if necessary to be sure that the culture was adapted to these conditions and that methanogenesis was coupled to growth.

**Analysis of proteins.** Electrode buffer contained (per liter) 3.09 g of Tris base (adjusted to pH 8.3 with HCl), 13.6 g of glycine, and 1 g of sodium dodecyl sulfate (SDS). Stacking-gel buffer contained (per liter) 15 g of Tris base (pH 6.8, adjusted with HCl) and 1 g of SDS. Separation gel buffer contained (per liter) 45.4 g of Tris base (adjusted to pH 8.8 with HCl) and 1 g of SDS. The electrophoretic analysis was performed at 15°C with an Epphorect slab gel electrophoresis unit (Haake Buchler Instruments, Inc., Saddle Brook, N.J.). The separation gel was prepared by dissolving 11 g of acrylamide and 0.275 g of bisacrylamide in 100 ml of the separation gel buffer and adding 100 μl of N,N,N',N'-tetramethylethylene diamine and 200 μl of a fresh ammonium persulfate solution (7.5%, wt/vol). The stacking gel was prepared by dissolving 4.5 g of acrylamide and 0.12 g of bisacrylamide in 100 ml of stacking-gel buffer and adding 162 μl of N,N,N',N'-tetramethylethylene diamine and 0.67 ml of 0.05% (wt/vol) fresh ammonium persulfate solution. Proteins were extracted from cells by suspending the cells in electrode buffer to which we added (per liter) 99 ml of glycerol, 12.5 g of SDS, and 11.2 ml of β-mercaptoethanol; approximately 40 ml of this solution was used per g of protein in the cell pellet. The suspension was heated in boiling water for 5 min, and protein was determined by the optical density at 640 nm of the protein solution diluted 40-fold with 10% (wt/vol) trichloroacetic acid (bovine serum albumin was used as a control). For each 1 ml of protein solution we added 0.16 ml of a solution containing 400 g of sucrose per liter and 2.5 g of bromothymol blue per liter; a volume of this mixture containing 15 to 40 μg of protein was added to each sample well. We applied a voltage of 90 V for 10 min to drive the proteins into the stacking gel. Then the voltage was reduced to 45 V while the samples passed through the stacking gel (approximately 1.5 h) and increased to 200 V for separation in the separation gel (approximately 4.5 h). Gels were fixed and stained overnight with Coomassie blue (a solution containing 0.5 g of Coomassie blue per liter, 250 g of isopropanol per liter, and 100 ml of glacial acetic acid per liter), washed with deionized water for 4 h, and destained and stored in 10% (vol/vol) glacial acetic acid. A 600-nm narrow-band-pass filter (catalog no. 165-2061; Bio-Rad Laboratories, Richmond, Calif.) was used to enhance resolution when the gel was photographed.

**Analytical techniques.** The amounts of methane were calculated from the headspace volumes in culture vessels and the partial pressures of methane. The partial pressure of methane was measured directly by maintaining samples at the pressure of the culture vessel during collection and quantifying methane by gas chromatography. We collected samples by filling a 10-μl loop with a gas sample by using a two-position Valco valve (Valco Instruments, Houston, Tex.). In position 1, one end of the loop was connected to a 0.5-ml dead volume on which a vacuum had been drawn. The other end of the loop was connected with 1.6-mm stainless-steel tubing to a syringe needle inserted into the headspace of the culture vessel. Thus, gas from the culture vessel flowed through the loop and into the 0.5-ml space, filling the loop with gas under pressure equal to that of the culture vessel. When the valve was turned to position 2, the loop was placed in the flow of carrier gas to a gas chromatograph equipped with a column of activated charcoal and a flame ionization detector.

The O₂ in groundwater samples was quantified by using an O₂ probe. Gram staining was performed by the Hucker method (7). We isolated DNA by using the method of Marmur (10), and W. B. Whitman determined the guanine-plus-cytosine content by high-performance liquid chromatography after enzymatic hydrolysis (12). We used epifluorescence microscopy with a type O2 filter set (Carl Zeiss, Inc., Thornwood, N.Y.) to distinguish colonies likely to be methanogenic by their blue-green epifluorescence.

For electron micrographs, cells were fixed in a cacodylate-buffered solution containing osmium tetroxide (10 g/liter) and glutaraldehyde (25 g/liter) (2).

**RESULTS AND DISCUSSION**

**Isolation.** A slurry of subsurface solids from near West Alkali Lake in Oregon was collected. The slurry appeared to be anoxic, based on sulfurous odors (perhaps of methylated sulfides) and measurement of O₂ in nearby multilevel wells, which suggested a lack of O₂ at 1.0 m below the water table. The temperature of the aquifer was 18°C. The sample was returned to the laboratory, and within 24 h of collection the sample was suspended and diluted into enrichment medium and into enrichment medium with trimethylamine replaced by the following substrates: 100 mM formate, 101 Pa of H₂, and 50 mM acetate. Methanogenesis occurred in trimethylamine-containing cultures inoculated with as little as 1 nl of the original slurry, but no significant quantities of methane were detected in media supplemented with other substrates.
When growth of the culture inoculated with 1 nl was complete, the culture was transferred to fresh media and maintained as an enrichment culture during isolation attempts. When site water was omitted from the enrichment medium, cultures failed to grow after several transfers unless KCl was included.

We used solid media in roll tubes for isolation of methanogens from this enrichment culture. Initially, cultures picked from epifluorescent colonies contained contaminants, so we included antibiotics (2 g of penicillin G per liter and 0.1 g of D-cycloserine per liter) in the roll tube media to inhibit the growth of nonmethanogens. After we obtained an apparently pure culture, the antibiotics were eliminated, and a single colony type was observed in two sequential roll tube isolations. The isolated culture was named strain WALIT\textsuperscript{T}.

When this organism was cultivated in MSHA medium, which is rich in yeast extract and peptones, no contaminants were detected. We also inoculated fluid thioglycolate medium and MSHA medium with air added, and no growth of contaminants was detected. Strain WALIT\textsuperscript{T} was deposited in the Oregon Graduate Institute Collection of Methanogenic Archaeobacteria as strain OGI 99\textsuperscript{T}.

**Morphology.** Surface colonies on solid media were circular, smooth, and tan and reached a diameter of 1 mm in 10 days (Fig. 1A). The cells comprising the colony were irregular coccoids (diameter, 1.0 to 1.5 \( \mu \)m) and existed as individual cells. In liquid culture, individual cells also occurred, but, especially during growth in medium containing low K\textsuperscript{+} concentrations, we observed many clumps containing 2 to 15 cells (and sometimes more). Individual cells and
clumps were sensitive to lysis in the presence of 1 g of SDS per liter, although clumps took longer to lyse. No outer membrane or typical gram-positive cell wall was seen in thin sections (Fig. 1B); the cells stained gram negative. The surface layer of the cells appeared to be protein (Fig. 1C).

**Physiology.** The cells produced methane as a major catabolic product. Stoichiometric amounts of methane were formed from trimethylamine, methanol, and dimethylsulfide (5 mM), but not from formate (100 mM), acetate (50 mM), or \( \text{H}_2\text{CO}_3 \). Cells grew only about one-fourth as rapidly when methanol rather than trimethylamine was present as the catabolic substrate. The presence of 101 Pa of \( \text{H}_2 \) in cultures growing on trimethylamine did not change the ultimate amount of methane formed. Because dimethylsulfide may be toxic (9), we compared methanogenesis in cultures supplemented with 5 mM dimethylsulfide, 5 mM trimethylamine, and 5 mM trimethylamine plus 5 mM dimethylsulfide; the cultures supplemented only with dimethylsulfide produced small amounts of methane, and those supplemented with 5 mM trimethylamine plus 5 mM dimethylsulfide produced significantly more methane than cultures supplemented with 5 mM trimethylamine alone.

The cells were strictly anaerobic, and motility was not observed in wet mounts in which cells were free in the liquid and not touching the slide or cover slip. No flagella were seen in electron micrographs. Measurement of specific growth rates under various conditions (Fig. 2) indicated that strain WALT is alkalophilic (pH optimum, 8.1 to 9.1), mesophilic, and halotolerant (good growth in medium containing up to 1.5 M Na\(^+ \)). \( \text{K}^+ \) was required, and \( \text{Mg}^{2+} \) was stimulatory (Fig. 2). Most rapid growth occurred with yeast extract, although a vitamin mixture (3) or Trypticase peptones could also support growth.

**DNA analysis.** The guanine-plus-cytosine content of the DNA was 40.9 ± 0.1 mol%.

**Taxonomy.** The physiology and morphology of strain WALT suggested that this organism is a member of the genus *Methanohalophilus*. Two other moderately halophilic genera have been described (*Methanohalobium* [22] and *Halomethanococcus* [20]), but we regard these names to be subjective synonyms of *Methanohalophilus* because the characteristics of these genera which have been described are very similar to those of *Methanohalophilus* because the characteristics of these genera which have been described are very similar to those of *Methanohalophilus* and *Methanohalophilus* has precedence. The characteristics (other than alkaliphily) of strain WALT are also similar to those of members of the genus *Methanococcoides*, although strain WALT is more halotolerant (Table 1); strain WALT grew well at Na\(^+ \) concentrations (1.2 to 1.4 M) at which *Methanococcoides methylutens* cannot grow (18). Also, *Methanococcoides methylutens* requires no organic growth factors and can grow in medium with its catabolic substrate, trimethylamine, as the sole organic addition. In contrast, strain WALT requires vitamins. Strain WALT is physiologically most similar to *Methanohalophilus zilinae* (6, 11) (Table 1), so we propose that strain WALT should be placed in the same genus. Strain WALT is different from *Methanohalophilus zilinae* WeN5T in that the former is halotolerant,
TABLE 1. Comparison of characteristics of some halophilic type strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Reference(s)</th>
<th>Guanine-plus-cytosine content (mol%)</th>
<th>Size (µm)</th>
<th>Na(^+) concn (M)</th>
<th>Temp (°C)</th>
<th>pH</th>
<th>Organic growth requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanohalophilus oregonense WAL1(^T)</td>
<td>None</td>
<td>41</td>
<td>1-1.5</td>
<td>0.48</td>
<td>35</td>
<td>8.6</td>
<td>8.2-9.2, Vitamins</td>
</tr>
<tr>
<td>Methanohalophilus mahii SLP(^T)</td>
<td>15, 16</td>
<td>49</td>
<td>1-2.5</td>
<td>1.2(^b)</td>
<td>37(^b)</td>
<td>7.5</td>
<td>7.0-7.8, ND</td>
</tr>
<tr>
<td>Methanohalophilus zhilinae WeN5(^T)</td>
<td>6, 11</td>
<td>38</td>
<td>0.8-1.5</td>
<td>0.75</td>
<td>45</td>
<td>9.3</td>
<td>8.6-9.6, Vitamins(^d)</td>
</tr>
<tr>
<td>Methanococcoides methylatus TMA-10(^T)</td>
<td>18</td>
<td>42</td>
<td>1-3</td>
<td>0.22</td>
<td>35</td>
<td>7.0</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(^a\) The range of conditions that support a growth rate that is at least 50% of the maximum growth rate.

\(^b\) Specific growth rates were not determined for this organism, so optimal conditions were inferred from the accumulated methane in culture vessels.

\(^c\) ND, Not determined.

\(^d\) Unpublished data.

whereas the latter is halophilic. Furthermore, Methanohalophilus oregonense WAL1\(^T\), having an optimum temperature higher than the maximum temperature at which strain WAL1\(^T\) can grow. Furthermore, an electrophoretic comparison of whole-cell proteins (Fig. 3) demonstrated major differences between the proteins of these two organisms, indicating that strain WAL1\(^T\) does not belong in the species Methanohalophilus zhilinae. Thus, we propose a new species, Methanohalophilus oregonense, with strain WAL1 as the type strain.

*Methanohalophilus oregonense* sp. nov. Liu and Boone. *Methanohalophilus oregonense* (or.e.gon.en’se. N. L. adj. oregonense, from Oregon, indicating the source type of the type strain) cells are irregular, nonmotile coccoids (diameter, 1 to 1.5 µm) which exhibit a gram-negative reaction. Coccoids may occur individually or in aggregates. Cells are sensitive to lysis by 0.2 g of SDS per liter. Trimethylamine, methanol, and dimethylsulfide are catabolic substrates, but H\(_2\)-CO\(_2\), formate, and acetate are not. Growth is most rapid with yeast extract in the medium, but vitamins or peptones support growth.

Surface colonies are approximately 1 mm in diameter after 10 days; they are circular, entire, smooth, and tan.

Growth is most rapid at 0.1 to 1.5 M Na\(^+\), and no growth occurs at 1.75 M Na\(^+\). Growth is rapid at pH 8.2 to 9.2, and growth occurs at pH values between 7.6 and 9.4. Growth is most rapid at 35 to 37°C, good growth occurs at 30 to 40°C, and no growth occurs at 10 or 45°C.

The guanine-plus-cytosine content is 40.9 ± 0.1 mol%, as determined by chromatographic analysis of nucleoside bases.

The type strain is strain WAL1 (= OGI 99 = DSM 5435), which was isolated from a slurry of subsurface solids from a saline, alkaline aquifer.

![FIG. 3. Electrophoretic comparison of whole-cell proteins of Methanohalophilus oregonense WAL1\(^T\) and related organisms. (A) For the photograph of the gel we used a 600-nm narrow-band-pass filter. (B) Locations and relative sizes of protein bands determined by visual observation of the gel.](image-url)
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LITERATURE CITED