Methanohalophilus zhilinae sp. nov., an Alkalophilic, Halophilic, Methylotrophic Methanogen

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Methanohalophilus zhilinae, a new alkaliphilic, halophilic, methylotrophic species of methanogenic bacteria, is described. Strain WeNST (T = type strain) from Bosa Lake of the Wadi el Natrun in Egypt was designated the type strain and was further characterized. This strain was nonmotile, able to catabolize dimethylsulfide, and able to grow in medium with a methyl group-containing substrate (such as methanol or trimethylamine) as the sole organic compound added. Sulfide (21 mM) inhibited cultures growing on trimethylamine. The antibiotic susceptibility pattern of strain WeNST was typical of the pattern for archaeobacteria, and the guanine-plus-cytosine content of the deoxyribonucleic acid was 38 mol%. Characterization of the 16S ribosomal ribonucleic acid sequence indicated that strain WeNST is phylogenetically distinct from members of previously described genera other than Methanohalophilus and supported the partition of halophilic methanogens into their own genus.

Methanogenesis occurs in sediments of some alkaline, saline lakes, as demonstrated by enrichment and isolation techniques. Methanogenic enrichment cultures from Big Soda Lake in Nevada catabolize methanol, trimethylamine, dimethylsulfide, and methane thiol (9). Only compounds which contain methyl groups are used as substrates; H2-CO2 is not used by isolates or in enrichment cultures. Some or all of these bacteria belong to a recently described genus, Methanohalophilus (16). In contrast, alkaliphilic isolates from nonsaline environments use only H2-CO2 for growth and methanogenesis (2, 3, 21). Previously (3), we reported the isolation from Bosa Lake of strain WeNST (T = type strain), the first methylotrophic, alkaliphilic methanogen isolated in axenic culture. In this paper this organism, which uses trimethylamine and methanol for growth and methanogenesis, is further characterized and is designated the type strain of Methanohalophilus zhilinae sp. nov.

MATERIALS AND METHODS

Inocula and media. Strain WeNST was isolated (3) from Bosa Lake in the Wadi el Natrun, Egypt, by using the techniques and media described below. The culture medium had the following composition (per liter of Milli-Q-deionized water, 5.9 mM): 3.0 g of Na,HCO3, 2.0 g of Na2CO3, 2.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 2.0 g of Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 40 g of NaCl, 1.0 g of NH4Cl, 3.5 g of MgCl2·6H2O, 3.0 g of MgSO4·7H2O, 1.0 g of KCl, 0.4 g of K2HPO4, 1 mg of resazurin, 10 ml of a trace minerals solution (4), 0.5 g of cysteine hydrochloride monohydrate, and 0.25 g of Na2S·9H2O. The gas phase was nitrogen, and the pH was 9.3. The enrichment medium contained 0.5 g of yeast extract, 0.5 g of Trypticase peptone, 250 g of NaCl, 0.5 g of Na2S·9H2O, and no cysteine hydrochloride monohydrate. The isolation medium (3) was the same as the enrichment medium except that the NaCl concentration was reduced to 120 g liter and 15 g of purified agar per liter was added.

Culture techniques. Anaerobic culture techniques of Hungate (7) were used. Inoculations were made by using syringes flushed with O2-free gas. Enrichment cultures were incubated at 37°C without shaking, and all other cultures were incubated at 45°C; the inoculum volume was 1.7%. The effects of pH, NaCl, and temperature (reported previously [3] and the effects of sodium sulfide were measured by adjusting the normal culture medium as required and calculating the growth rate early in the exponential phase. Specific growth rates were determined by measuring the methane accumulation rates in the culture vessels; the contribution of inoculum was considered when the specific growth rate was calculated (17). When the growth rates at various pH values were measured (3), a HCO3−-CO2− buffer was used; media were prepared with 5 g of Na2CO3 per liter, and CO2 was added to obtain the desired pH.

Determination of 16S rRNA partial sequences. We purified and partially sequenced 16S ribosomal RNA (rRNA) by using the reverse transcriptase procedure of Lane et al. (11). The procedures of Lane et al. were followed exactly. This approach employs 16S rRNA-specific oligodeoxynucleotide primers in conjunction with dideoxynucleotide sequencing by reverse transcriptase (Seikagaku America). The deoxyribonucleic acid primers were made by beta-cyanoethyl phosphoramidite chemistry (18), using an automated procedure (Bioresearch model 8700). The particular primers used in this study (International Union of Biochemists nomenclature; Escherichia coli 16S rRNA positions are indicated in parentheses) were ATGCCCTCCGTCGT (357), TTTGARTTTMCTTAAC (1046), and TTTGARTTTMCTTAAC (1510).

Analytical techniques. Lysis by sodium dodecyl sulfate and susceptibility of cultures to antibiotics were tested as previously described (13). The guanine-plus-cytosine content of the deoxyribonucleic acid was determined by the buoyant density method, and methane was determined by gas chromatography (1). The total dissolved solids content of the lake...
**FIG. 1.** Extensive partial sequences. Extensive partial sequences of strain WeNST are shown aligned with the known complete sequence of *H. volcanii* (5). The sequences were aligned according to primary sequence homology, with minor alignment adjustments to ensure conformity with known secondary structure features of 16S rRNA (6). A, Adenine; U, uracil; C, cytosine; G, guanine. Lowercase letters indicate uncertainty. The following other designations indicate ambiguity: n, unknown base; x, purine; y, pyrimidine; k, guanine or uracil; s, guanine or cytosine; m, adenine or cytosine. Ambiguities may be due to heterogeneity in the rRNA population or to inherent limitations of the method. Dots are used to indicate positions known to be deleted in the sequence. Unsequenced regions are left blank.
water was determined by drying a sample of filtered lake water to constant weight at 105°C.

RESULTS AND DISCUSSION

The isolation and partial characterization of strain WeNST (= DSM 4017T) have been described previously (3).

Lake and sediment characteristics. The sediments of the lake were black and smelled of hydrogen sulfide; the lake water was reddish. The pH of the lake water was 9.7. Its specific conductance was greater than 0.50 mS m⁻¹, and the total dissolved solids content was 245 g/liter. The reddish color may have been due to the presence of phototrophic, halophilic, sulfur-oxidizing bacteria, such as *Ectothiorhodospira*, which have been isolated previously from lakes in the Wadi El Natrun (8).

Characteristics of WeNST. In addition to trimethylamine and methanol (3), strain WeNST used dimethylamine and monomethylamine as methanogenic substrates when they were provided at concentrations of 20 mM. Dimethylsulfide (5 mM) supported growth and methanogenesis as a sole catabolic substrate, but in medium containing 20 mM dimethylsulfide methane production was inhibited. In such medium, little methane was produced even when 20 mM trimethylamine was also present.

Wet-mount slides of cells were prepared in an anaerobic chamber and were sealed with petroleum jelly-paraffin (1:1, vol/vol). When these slides were viewed microscopically outside the chamber, no motility was observed. The presence of flagella was not directly determined, and the possibility of motility too weak to be observed was not excluded.

Growth occurred in mineral medium at about one-third (specific growth rate, 0.04 h⁻¹) the rate of growth in complex medium. Cultures in mineral medium supplemented with yeast extract (0.2%, wt/vol), Trypticase peptone (0.2%, wt/vol), or rumen fluid (5%, vol/vol) grew as well as cultures in normal medium; acetate (5 mM) was not stimulatory. Strain WeNST produced methane in medium containing elevated sulfide concentrations, but this may have been uncoupled from growth. When strain WeNST was inoculated into mineral medium containing 21 mM sulfur (H₂S + S²⁻), the methanogenic rate shortly after inoculation was similar to the rate of controls containing 1.0 mM sulfide (the normal medium concentration). However, the methanogenic rate did not increase logarithmically, and after subsequent transfer into identical media (with elevated sulfide concentrations) cultures took longer to complete methanogenesis (twice as long at a concentration of 21 mM and four times as long at a concentration of 42 mM). After the next transfer the cultures containing 21 and 42 mM sulfide did not grow or produce methane.

The susceptibility of strain WeNST to various antibiotics (each at a concentration of 100 mg/ml) was typical for methanogenic bacteria. Inhibitors of cell wall synthesis (penicillin G, ampicillin, carbenicillin, and cycloserine) were not inhibitory; ribosome-affecting compounds (chloramphenicol and tetracycline) completely inhibited growth. The guanine-plus-cytosine content of the deoxyribonucleic acid, measured by the buoyant density method, was 38 mol%.

16S rRNA sequence characterization. Sequence data were obtained for 918 of the estimated 1,450 nucleotide positions in 16S rRNA from strain WeNST (Fig. 1). These data convincingly support the separation of strain WeNST from all other methanogens and extreme halophiles whose 16S rRNA sequences or oligonucleotide catalogs have been determined. (The rRNA of *Methanohalophilus mahii* SLP (16) has not been characterized.) The levels of sequence similarity were 78.8% with *Methanobacterium formicicum*, 81.8% with *Methanospirillum hungatei*, and 78.8% with *Haloferax volcanii*.

Taxonomy. Halophilic, methylotrophic, cocoid methanogens are consistent with the descriptions of the following four currently recognized genera: *Methanolobus* (10), *Methanococoides* (20), *Methanosarcina* (*Methanosarcina acetivorans*) (19), and *Methanohalophilus* (16). A fifth genus, *Methanococcus*, contains a halophilic, methylotrophic, cocoid species (22), but the description of this genus specifically precludes the inclusion of methylotrophic organisms (12). The genera *Methanosarcina*, *Methanolobus*, and *Methanococoides* contain species which are only weakly halophilic. *Methanohalophilus* is the only genus whose description (16) includes moderate halophiles. Thus, strain WeNST appears to belong in the genus *Methanohalophilus*.

At least nine other methanogenic isolates have been described, which, as a group, differ significantly from other genera and fall within the description of *Methanohalophilus* (16). These isolates may be separated from less halophilic genera by the requirement of the former for much higher concentrations of salt for rapid growth and generally higher temperature optima. These isolates are *Methanococcus halophilus* Z-7982 (= DSM 3094) (22), *Methanohalophilus mahii* SLP (= ATCC 35705) (15, 16), strain SF1 (= DSM 3243) (13), five strains described by Zhilina (23) (strains Z-7301, Z-7302, Z-7303, Z-7305, and Z-7403), and strain WeNST. The major physiological distinction between strain WeNST and all of these other strains is its extremely high pH requirement for optimum growth.

Description of *Methanohalophilus zhilinae* sp. nov. *Methanohalophilus zhilinae* (zhil'in'ae. n. L. gen. n. *zhilinae* of Zhilina, named for Tatjana N. Zhilina in recognition of her many contributions to the biology and ecology of methanogens, especially methanogenesis in saline and hypersaline environments. Irregular cocci, 0.75 to 1.5 μm in diameter, occurring individually and occasionally in clumps and tetrad. Very susceptible to lysis by sodium dodecyl sulfate. Gram stain results are negative. Colonies in roll tubes are 0.2 to 0.4 mm within 7 days, yellowish tan, smooth, convex, opaque, and circular with entire margins. Methyllamines, methanol and other methyl group-containing substrates (but not acetate) are used for growth and methanogenesis; H₂, CO, and formate are not used. Yeast extract, Trypticase peptone, and rumen fluid are stimulatory but not required. Trimethylamine may serve as a sole carbon and energy source.

Grows in medium containing 0.2 to 2.1 M NaCl; optimal growth in the presence of 0.7 M NaCl. Most rapid growth occurs near pH 9.2 and 45°C.

The guanine-plus-cytosine content of the deoxyribonucleic acid is 38 mol%.

Habitat: alkaline, saline lake sediments. Type strain: WeNST (= DSM 4017).

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