**Methanobacterium alcaliphilum** sp. nov., an H₂-Utilizing Methanogen That Grows at High pH Values

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Four strains of alkaliphilic methanogens (strains WeN1, WeN2, WeN3, and WeN4T [T = type strain]) previously enriched and isolated from sediments of four low-salt, high-pH (pH 8.3 to 9.3) lakes in the Wadi el Natrun of Egypt were further characterized. These organisms were H₂-oxidizing, CO₂-reducing, rod-shaped bacteria which grew best at high pH values (strains WeN1, WeN2, and WeN4T preferred pH 8.4 and grew at pH values up to 9.9; strain WeN3 grew best at pH 7.8 and grew at pH values up to 9.2). Each strain required sulfide as a sulfur source and was resistant to elevated concentrations (growth rates in the presence of 0.5% Na₂S - 9H₂O were one-third to one-half the maximum observed rates). The guanine-plus-cytosine content of the deoxyribonucleic acid of strain WeN4T was 57 mol%. A new species of methanogen, *Methanobacterium alcaliphilum*, is described; strain WeN4 (= DSM 3387) is the type strain.

Most previously studied methanogens were isolated from habitats with pH values near neutrality, and such pH values support the best growth of these microbes. Recently, several alkaliphilic strains were isolated, including the thermophilic organism *Methanobacterium thermoalcaliphilum* (3) and four mesophilic isolates from the Wadi el Natrun in Egypt (D. R. Boone, S. Worakit, I. M. Mathrani, and R. A. Mah, Syst. Appl. Microbiol., in press).

We report here further characterization of the four mesophilic strains, which belong to a new species of bacterium, *Methanobacterium alcaliphilum* sp. nov.

**MATERIALS AND METHODS**

**Inocula and media.** Sediment samples were collected from four Egyptian lakes of the Wadi el Natrun (Boone et al., in press). The culture media used contained (per liter of deionized water) 10 g of NaHCO₃, 2.0 g of yeast extract, 2.0 g of Trypticase peptone, and L-cysteine hydrochloride, 0.1 g of MgCl₂·6H₂O, 0.02 g of CaCl₂·0.4 g of K₂HPO₄, 1 mg of resazurin, 0.1 mg of H₂SeO₃, 1 mg of FeSO₄·7H₂O, 1.5 mg of CoCl₂·6H₂O, 1 mg of ZnCl₂, 0.1 mg of H₃BO₃, 0.1 mg of NaMoO₄·2H₂O, 0.2 mg of CuCl₂·2H₂O, 0.2 mg of NiSO₄·6H₂O, 0.4 mg of AlCl₃·6H₂O, and 5 mg of disodium ethylenediaminetetraacetic acid dihydrate. The gas phase was 100% N₂. All ingredients except NaHCO₃, yeast extract, Trypticase peptone, and L-cysteine hydrochloride were dissolved in 1,020 ml of deionized water, which was boiled under a stream of O₂-free nitrogen. Trypticase peptone, yeast extract, and L-cysteine hydrochloride were added; for solid media, 18 g of purified agar was also added. Boiling was continued until the volume was reduced to 1,000 ml. The medium was cooled, and O₂-free NaHCO₃ was added. The NaHCO₃ was rendered O₂-free by storage in an anaerobic chamber or in a sealed tube flushed with N₂ gas. The medium was distributed to serum tubes, which were sealed with butyl rubber stoppers secured with aluminum crimp closures and autoclaved. The final pH was 8.4. Additions of soluble substrates were made from sterile, O₂-free stock solutions. H₂ was added immediately after inoculation by pressurizing the atmosphere in the tubes to 170 kPa (the total H₂ partial pressure was 68 kPa). The medium used for enrichment (Boone et al., in press) was made by decreasing the Trypticase peptone and yeast extract concentrations to 0.5 g of each per liter and by omitting L-cysteine hydrochloride. Media of different pH values were prepared by adding O₂-free, sterile 1 M solutions of HCl or NaOH to tubes.

**Culture techniques.** In this study and a previous one (Boone et al., in press) the culture techniques of Hungate (4) were used, with some of the modifications described by Balch and Wolfe (1). Cultures were incubated at 37°C; those with added H₂ were incubated in a shaker. As H₂ was used, it was periodically replaced by represurizing the tubes with a mixture of H₂ and CO₂ (4:1).

The effect of the pH value of the medium on growth was determined (Boone et al., in press) by measuring the specific growth rate. The culture medium was initially adjusted to various pH values, and growth was monitored during the early phase when the pH of the medium increased by no more than 0.2 pH unit. Media adjusted to pH values above 8.3 were prepared with 50 mM 2-cyclohexylaminoethanesulfonate buffer.

**Analytical methods.** Optical density was determined with a Spectronic 70 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). Methane was analyzed by gas chromatography with thermal conductivity detection. Growth rates were determined from the exponential increase in the sum of inoculum-produced methane and the methane accumulated in culture vessels (6). Often these growth rates were corroborated by optical density measurements; these latter growth rates were always similar to those calculated from methane production but were less precise. A Zeiss Universal Research microscope was used for epifluorescence microscopy. Deoxyribonucleic acid was isolated by the method of Marmur (5) and was analyzed by the buoyant density method with CsCl gradients (7).

**Radioisotopic techniques.** To determine the ability of cells to assimilate acetate, 0.54 μCi of [U-14C]acetate and 15 μmol of unlabeled acetate were added per 5 ml of medium (the
WeN2 incorporated yeast extract and 3 mM acetate and found that strain WeN1 partially characterized as described elsewhere (Boone et al., in press). Strain WeN2 without added sulfide was completed, the cultures did not grow in fresh media. The percentage of incorporation was calculated as the weight of the control filters. The radioactivity on the filters was determined by counting in scintillation vials with 15 ml weight in a vacuum oven at 105°C. Uninoculated-medium was determined by counting in scintillation vials with 15 ml trichloroacetic acid and then with 10 ml of water and 10 ml of 3 mM sodium acetate. The filters were dried to a constant rate.

The morphology and substrate range of the four strains studied place them in the genus Methanobacterium. However, these four strains differ from previously described species of the genus Methanobacterium by their lack of immunological cross-reactions, their high pH range for growth, and the high guanine-plus-cytosine contents of their deoxyribonucleic acids (Boone et al., in press). Unlike other species of Methanobacterium, our four strains stained gram negative; two other isolates ("Methanobacterium thermoaggregans" [2] and "Methanobacterium thermoacaliphilum" [3]) appear to be gram-negative bacteria which belong to the genus Methanobacterium. We propose a new species, Methanobacterium alcaliphilum, for our isolates, with strain WeN4 as the type strain.

There were significant differences between strain WeN3 and strains WeN1, WeN2, and WeN4T, including a slightly lower temperature optimum (Boone et al., in press), a lower pH optimum (Boone et al., in press), a lower rate of acetate incorporation, and an ability to grow for several transfers in mineral medium (Boone et al., in press). However, the similarities between strain WeN3 and the other strains indicate that at present the description of M. alcaliphilum should be broad enough to include all four strains.

We propose the species description given below.

**Methanobacterium alcaliphilum** sp. nov. (al.ca.li.phi lum. M.L. alcali alkalii [from Arabic al end; galiy soda ash]; Gr. adj. philum loving; M.L. adj. alcaliphilum liking alkaline media). Long rods, 0.5 to 0.6 by 2 to 25 μm, occurring individually or in pairs, more rarely in short chains or filaments. Gram negative. Nonmotile. Colonies grow to 1.0 to 1.5 mm in diameter. Colonies are yellowish to cream colored, smooth, opaque, raised, convex, and circular with entire margins. Subsurface colonies are cream colored, small (diameter, 0.2 mm) irregular spheroids. H₂-CO₂ is the sole substrate for growth and methanogenesis. Growth factors present in Trypticase peptone or yeast extract are required for growth.

Optimal growth occurs between pH 8.1 and 9.1 and near 37°C.

The quanine-plus-cytosine content of the deoxyribonucleic acid is 57 mol%.

The type strain is strain WeN4 (= DSM 3387).

**RESULTS AND DISCUSSION**

The four strains used in this study were isolated and partially characterized as described elsewhere (Boone et al., in press). Strains WeN1 (= DSM 3457), WeN2 (= DSM 3458), WeN3 (= DSM 3459), and WeN4T (= DSM 3387) (T = type strain) were maintained by weekly transfers of 2 ml of culture into 20 ml of liquid medium in serum vials. Microscopic examination of wet mounts revealed no motility.

**Acetate incorporation.** Acetate was not required for growth and did not relieve the requirement for Trypticase peptone and yeast extract. We repeated earlier tests (Boone et al., in press) of acetate assimilation in the presence of yeast extract and 3 mM acetate and found that strain WeN1 incorporated 7.0% of its cell carbon from acetate; strain WeN2 incorporated 8.5%; strain WeN3 incorporated 0.9%; and strain WeN4T incorporated 11%.

**Sulfide requirement and toxicity.** Figure 1 shows the effect of sulfide concentration on the growth rates of cells. In medium without sulfide (but containing cysteine, Trypticase peptone, and yeast extract as potential sulfur sources), all strains grew after the first transfer. However, after growth was completed, the cultures did not grow in fresh media without added sulfide (5%, vol/vol, transfer), indicating that sulfur sources present as medium components could not replace sulfide.

**Taxonomy.** The morphology and substrate range of the four strains studied place them in the genus Methanobacterium. However, these four strains differ from previously described species of the genus Methanobacterium by their lack of immunological cross-reactions, their high pH range for growth, and the high guanine-plus-cytosine contents of their deoxyribonucleic acids (Boone et al., in press). Unlike other species of Methanobacterium, our four strains stained gram negative; two other isolates ("Methanobacterium thermoaggregans" [2] and "Methanobacterium thermoacaliphilum" [3]) appear to be gram-negative bacteria which belong to the genus Methanobacterium. We propose a new species, Methanobacterium alcaliphilum, for our isolates, with strain WeN4 as the type strain.


