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Isolation and Characterization of a Fast-Growing, Thermophilic *Methanobacterium* Species

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A thermophilic, autotrophic methanogen (strain CB12, DSM 3664) was isolated from a mesophilic biogas digestor. This bacterium used H_2-CO_2 or formate as a substrate and grew as short rods, sometimes in pairs and in crooked filaments. Motility was not observed. Its optimum temperature (56°C) was lower than that of other thermophilic members of the genus *Methanobacterium*. The maximum observed specific growth rate was 0.564 h^{-1} (74-min doubling time).

Two recent studies (10, 11) reported the isolation of *Methanobacterium* strains with characteristics similar to those of *Methanobacterium thermoautotrophicum* (8) except that these strains were able to catabolize formate. The name "*Methanobacterium thermoformicicum*" was proposed for one of these isolates (10). In this note, we report on a new strain, CB12, which appears to be more similar to "*M. thermoformicicum*" than to *M. thermoautotrophicum*.

Modified (7) anaerobic techniques of Hungate were used in this study. A sludge sample was collected from a small mesophilic biogas plant located in Chengdu, China, which was fed a mixture of swine waste and human feces. The sample was quickly transported to the laboratory. Thermophilic H_2-CO_2- and formate-utilizing methanogens were enriched by addition of 1 ml of the sludge sample to 20 ml of medium in a serum vial; the culture was incubated at 55°C without shaking. The medium used for enrichment and isolation contained the following, per liter of distilled water: KH_2PO_4, 0.4 g; K_2HPO_4, 0.4 g; NH_4Cl, 1.0 g; NaCl, 1.0 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; KCl, 1.0 g; sodium acetate, 1.0 g; sodium formate, 10.0 g; yeast extract, 2.0 g; Trypticase peptone (BBL Microbiology Systems, Cockeysville, Md.), 2.0 g; L-cysteine hydrochloride, 0.5 g; $Na_2S \cdot 9H_2O$, 0.5 g; $NaHCO_3$, 1.0 g; resazurin, 1.0 mg; vitamin solution (6), 10 ml; and mineral solution (6), 10 ml. After CH_4 production subsided, 1 ml of the enrichment culture was transferred to fresh medium, which was incubated until CH_4 production stopped. On the third such successive transfer, 2 g of penicillin per liter was included in the medium to minimize growth of nonmethanogens. After CH_4 production ceased, the culture was serially diluted and inoculated into antibiotic-free roll tube medium (containing 20 g of agar per liter). Colonies were observed by epifluorescence microscopy; epifluorescent colonies were also examined by using a dissecting microscope and the naked eye. The colonies were circular, yellowish grey, smooth, moist, translucent, and raised with entire edges. They were the most numerous epifluorescent colony type and were 0.5 to 1 mm in diameter after 5 days. Such colonies were picked and subcultured in antibiotic-free medium until a single colony type was obtained. Cells were 0.4 to 0.6 μm wide and 2.5 to 6 μm long, with some filaments longer than 10 μm. Motility was not observed during microscopic examination of wet mounts.

Cells were gram positive and rod shaped (Fig. 1). During growth at 60°C, cells were 0.4 to 0.6 μm wide and 2.5 to 6.0 μm long. Some filaments were seen.

After isolation, cells were cultivated in medium with Trypticase peptone, yeast extract, and CO_2-HCO_3^- buffer (7) prepared with 3.6 g of $NaHCO_3$ per liter and N_2-CO_2 (7:3) gas phase. Medium prepared and sealed in vials at room temperature had a pH of 7.05, but after equilibration at 60°C the pH was 7.4. Cells grew and produced methane from formate (40 mM) or H_2 (68-kPa overpressure, via a needle through the stopper, was added after inoculation) but not from acetate (50 mM), methanol (40 mM), trimethylamine (20 mM), or monomethylamine (20 mM).

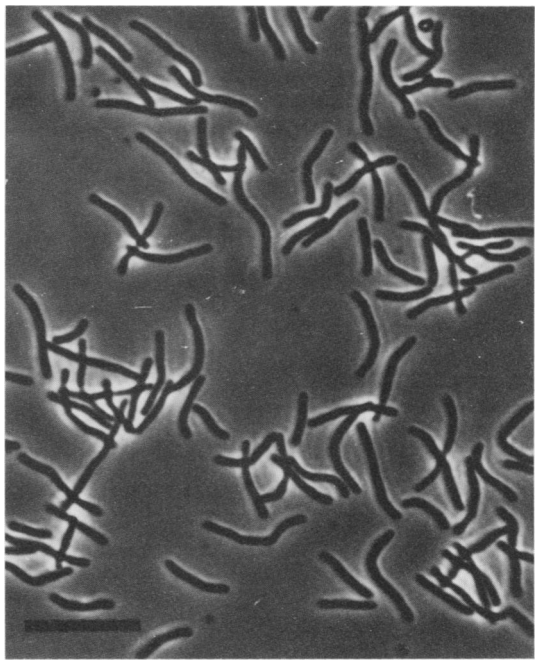

FIG. 1. Phase-contrast micrograph of strain CB12 grown at 55°C and pH 7.4 (bar = 10 μm).

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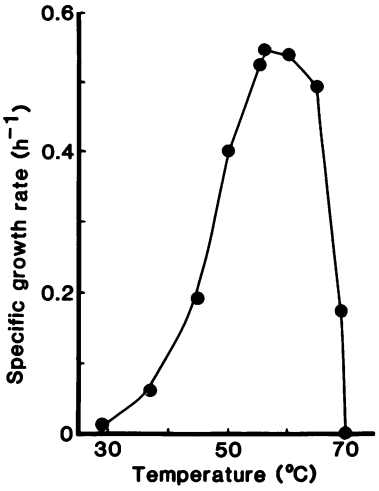

FIG. 2. Growth rate of strain CB12 grown in medium at pH 7.4 at various temperatures.

The growth rate was determined at 60°C in medium with H_2-CO_2 as the substrate. Medium at 60°C was inoculated with late-logarithmic-phase cells (2% [vol/vol]), pressurized to 169 kPa with 100% H_2 and incubated on a shaker at 60°C. After 5 h and each 1 h thereafter, cultures were repressurized to 169 kPa with H_2-CO_2 (3:1) to replenish the substrate and maintain pH. Vials (119 ml [total volume]) with 50 ml of medium were used for the determination of growth rate by measuring methane production. Growth rate was calculated by plotting the log of the sum of cumulative methane produced plus methane produced by the cells of the inoculum during its growth (4). Growth of parallel cultures in serum tubes was measured by optical density at 600 nm. Methane production measurements indicated growth rates of 0.542 h^{-1} (doubling time of 77 min) on H_2 and 0.551 h^{-1} on formate. Absorbance determinations indicated similar growth rates of 0.545 h^{-1} on H_2 and 0.449 h^{-1} on formate. The optimum growth temperature was between 55 and 65°C (Fig. 2); the fastest growth occurred at 56°C, lower than that of either *M. thermoautotrophicum* ΔH or the two other formate-utilizing, thermophilic methanogens (10, 11). Growth rate in mineral medium was also determined. Trypticase peptone, yeast extract, and cysteine were omitted, and the sulfide concentration was doubled. After four transfers (2% [vol/vol]) in mineral medium with H_2-CO_2 as the substrate, cultures had a growth rate of 0.567 h^{-1} (0.562 h^{-1} determined from optical density). The growth rate of strain CB12 was much faster than that reported for *M. thermoautotrophicum* ΔH (specific growth rates of 0.14 h^{-1} [8], 0.22 h^{-1} [4], and 0.32 h^{-1} [1] were reported) or Marburg (specific growth rate of 0.43 [1]).

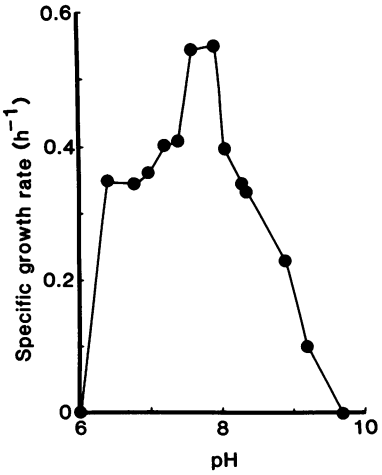

FIG. 3. Growth rate of strain CB12 in medium at 60°C at various pH values.

Optimum pH for growth was determined by measuring the growth rate in medium adjusted to various pH values (Fig. 3). pH values were adjusted by changing the concentration of CO_2 in the gas phase. Media below pH 5.4 were prepared with 100% CO_2, and the pH was adjusted with 1 M HCl. Media with pH values above 7.5 contained 10 mM 2-(cyclohexylamino) ethanesulfonate buffer. The pH values of media were determined at an incubation temperature of 60°C; all cultures were pressurized with H_2 (169 kPa) after inoculation and frequently repressurized with H_2-CO_2 (3:1) during growth. The pH value during growth never varied by more than 0.2, usually by less than 0.05. A logarithmic increase in methane production was observed in all cultures grown in medium with pH values between 6.4 and 9.2 (Fig. 3).

On the basis of morphology and autotrophy, strain CB12 resembles *M. thermoautotrophicum* more closely than other recognized species (8, 9). It also resembles *M. formicicum*; at least one strain of *M. formicicum*, strain JF-1 (5), has a similar temperature optimum. However, like two other recently isolated strains (10, 11), it differs from both *M. thermoautotrophicum* and *M. formicicum* by having a much more rapid growth rate. The optimum temperature for growth of strain CB12 is lower than that of these strains, and the minimum temperature for growth is also lower.

Although strain CB12 has the fastest reported growth rate of methanogens capable of growing in medium with low concentrations of salt, *Methanococcus thermolithotrophicus* SN1 (2) and *Methanococcus jannaschii* JAL-1 (3) are halophilic methanogens with doubling times of 55 and 26 min, respectively.

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ADDENDUM

The name *Methanobacterium thermoformicicum* has been validated (Int. J. Syst. Bacteriol. **36**:489, 1986), and strain CB12 appears to be a member of this species.

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