Isolation and Characterization of a Novel Thermophilic Methanosaeta Strain

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A novel thermophilic acetotrophic Methanosaeta strain was isolated from a thermophilic anaerobic digest or by using acetate enrichment and serial dilution in the presence of vancomycin and neomycin. This isolate, designated Methanosaeta sp. strain P, resembled Methanosaeta sp. strain CALS-1 morphologically; however, it occasionally formed filaments longer than 100 μm and exhibited autofluorescence. The content of coenzyme F₄₂₀ was much higher than that of Methanosaeta reference strains, and coenzyme F₄₂₀ with four glutamyl residues on the side chain was the predominant component. Furthermore, a comparative analysis of the antigenic fingerprint of strain P with the fingerprints of reference organisms showed that this isolate was not related antigenically to the reference methanogens, including Methanosaeta sp. ("Methanothrix") strain CALS-1 and Methanosaeta concilii ("Methanothrix soehngenii") Opfikon. Strain P formed visible colonies in a deep agar medium when high cell concentrations were present. However, transfer of a colony into liquid medium resulted in no growth. Strain P could utilize only acetate as a sole carbon and energy source. The optimum temperature and optimum pH for methanogenesis were near 55°C and 6.7, respectively. The specific methane formation rate μCH₄ under optimum conditions was 0.47 day⁻¹, and the doubling time was 1.49 days. The DNA base composition was 52.7 mol% guanine plus cytosine.

Acetate is a major intermediate in the anaerobic digestion of organic compounds, and conversion of acetate to methane is one of the rate-limiting reactions. Among the anaerobes, only some methanogens and sulfate-reducing bacteria can utilize acetate as a carbon and energy source. Of these microorganisms, members of the genus Methanosaeta ("Methanothrix") appear to be predominant and play an important role as acetate-consuming methanogens in anaerobic digestors, especially in up-flow anaerobic sludge blankets (6). Barker described a filamentous acetate-utilizing methanogen named Methanobacterium soehngenii about 50 years ago (2), but for many years no axenic cultures of Methanosaeta strains were obtained because of difficulties in cultivation (i.e., slow growth or no colony formation).

Methanosaeta sp. was first described as an "acetate organism" by Zehnder et al., and these authors designated their organism Methanothrix soehngenii Opfikon (11, 28). After this description, several other mesophilic Methanosaeta strains were isolated (8, 19, 26). Very recently, Patel and Sprott, who purified Methanothrix concilii GP6 (19), proposed that the genus Methanothrix should be changed to the genus Methanosaeta because the genus Methanothrix is nomenclaturally invalid according to the International Code of Nomenclature of Bacteria (13, 20). On the basis of the proposal by Patel and Sprott (20) and Touzel et al. (26), the mesophilic strains previously described (11, 12, 19, 26) should be assigned to the species Methanosaeta concilii.

A thermophilic strain of the genus Methanosaeta ("Methanothrix") was first enriched from thermal lake mud and other sources by Nozhevnikova et al. (17, 18). Recently, Zinder et al. obtained a pure culture of thermophilic Methanosaeta sp. strain CALS-1 from a thermophilic (58°C) anaerobic digestor (30, 31). However, details of the physiological and biochemical characteristics of this organism have not been described well. Therefore, the study of the genus Methanosaeta is in its infancy compared with the study of other methanogens, and thus much knowledge remains to be accumulated.

In the field of anaerobic digestion, there has been increased interest in high-temperature digestion because it is advantageous to obtain a higher reaction rate in the conversion of organic waste to methane than the rate that occurs in mesophilic processes. To elucidate the ecological, morphological, and physiological aspects of the role of thermophilic Methanosaeta strains in high-temperature digestion, we attempted to isolate a potent thermophilic Methanosaeta strain and obtained a novel strain from a thermophilic anaerobic digestor. In this paper we describe the isolation and characterization of a thermophilic isolate belonging to the genus Methanosaeta and discuss several novel aspects of our isolate.

MATERIALS AND METHODS

Microorganisms. Methanobacterium thermoautotrophicum DSM1053 and DSM2133, Methanosarcina barkeri DSM800, and Methanosaeta concilii DSM2139 ("Methanothrix soehngenii" Opfikon) and DSM2671 (=GP6) were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany. Methanosaeta concilii Uₐ and Pₘ were recently isolated from mesophilic anaerobic sludge digestors (12) by using a serial dilution technique (28) in our laboratory; these strains still contained a very small number of contaminants.

Media and growth conditions. The culture medium used for isolation and maintenance of stock cultures was medium 334 (5) of the Deutsche Sammlung von Mikroorganismen, which was first described by Touzel and Albagnac (25). Slight modifications were made during preparation; sodium acetate was added to a final concentration of 80 mM, and a vitamin solution was prepared as described by Balch et al. (1). Unless otherwise stated, all cultivations were carried out at 55°C in 125-ml serum vials containing 30 ml of medium or in 1,000-ml bottles containing 500 ml of medium under an atmosphere containing N₂ and CO₂ (80/20, vol/vol). The

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serum vials were closed with butyl rubber stoppers that were sealed with aluminum crimping.

*Methanobacterium thermoautotrophicum* DSM2133 and DSM1053 were cultivated at 60°C in 1,000-ml bottles containing 500 ml of medium of 310 mg of the Deutsche Sammlung von Mikroorganismen (5), except that the fatty acid mixture was excluded. Mixed gas (H2-CO2, 80:20, vol/vol) was supplemented at appropriate intervals, and the liquid and gas phases were mixed vigorously with a magnetic stirrer during cultivation.

*Methanosarcina barkeri* DSM800 was cultivated at 37°C in 1,000-ml bottles containing 500 ml of medium 119 of the Deutsche Sammlung von Mikroorganismen (5).

*Methanoseta concilii* DSM2139 ("Methanothrix soehngenii" Opfikon), DSM3671. U, and P, were cultivated at 37°C under the same conditions as strain P1.

**Microscopy.** An exponential-phase culture was observed by phase-contrast microscopy and epifluorescence microscopy, a type BP405 filter was used as the exciter filter and a type Y455 filter was used as the suppression filter.

**Analytical methods.** The amount of methane produced was determined by reversed-phase high-performance liquid chromatography (HPLC) (Shimadzu model LC-6A system) supplemented with fluorometric detection; our system was based on the system described by van Beelen et al. (27) and Gorris et al. (9, 10). For the fluorometric determinations, coenzyme F420 was detected by emission at 470 nm after excitation at 400 nm. Four types of coenzyme F420 (two to five glutamyl residues on the side chain) were separated from each other by this procedure. An authentic standard of coenzyme F420 with two glutamyl residue on the side chain was purified from *Methanobacterium thermoautotrophicum* DSM2133 by using the methods of Schoenheit et al. (23) and Eichler et al. (7).

**Antigenic fingerprint.** The antigenic fingerprint was determined as described previously by performing an indirect immunofluorescence and quantitative slide immunoenzymatic assay (14-16). We used the following 18 antibody probes, which were selected from a collection of 33 heterologous S-probes of reference methanogens: *Methanobacterium formicicum MF*; *Methanosarcina barreki MS*; *Methanobacterium bryantii MoH*; *Methanobacterium bryantii MoHG*; *Methanosarcina barkeri R1M3*; *Methanosipillium hungatei JF1*; *Methanobrevibacter arboriphilus DH1*; *Methanobacterium thermoautotrophicum GCI*; *Methanobacterium thermoautotrophicum DH*; *Methanosarcina Barkeri 227*; *Methanosarcina maezi S6*; *Methanosarcina Barkeri W*; *Methanosarcina thermophila TM1*; *Methanobrevibacter arboriphilus AZ*; *Methanobrevibacter arboriphilus DC*; *Methanothermus fervidus V24S*; *Methanoseta concilii ("Methanothrix soehngenii") Opfikon*; and *Methanoseta sp.* ("Methanothrix sp.") strain CALS-1. The levels of antigenic relatedness between the new isolate and the reference methanogens were determined by comparing the antigenic fingerprints, using a reference table (14).

**Determination of DNA base composition.** DNA was extracted and purified by using the methods of Beji et al. (3) and Saito and Miura (22). A brief sonication (10 kW, 5 s) was used after alkaline sodium dodecyl sulfate treatment to get a better yield. The purified DNA was hydrolyzed with P1 nuclease (GC kit; Yamasa Shoyu Co.) followed by alkaline phosphatase (from *Escherichia coli*; Wako Pure Chemicals Industry, Ltd.) as described by Tamaoka and Komagata (24). The guanine-plus-cytosine content was determined by reversed-phase HPLC (Shimadzu model LC-6A system). Separation was achieved at 50°C by using a flow rate of 1 ml/min, a column of YMC-Pak AQ-312 (Yamamura Chemical Institute Co.,) and 5% methanol in 10 mM phosphate buffer (pH 3.5) as the mobile phase. Each deoxyribonucleoside was detected by determining and an equimolar mixture of four deoxyribonucleosides was used as the standard.

**RESULTS**

**Isolation.** Digested sludge that was obtained from a thermophilic (55°C) anaerobic digestor in our laboratory was inoculated into acetate medium containing 100 µg of vancomycin per ml and was enriched at 55°C by consecutive transfers, using 10% (vol/vol) inocula. After 10 transfers at 2-week intervals, cells resembling the cell of thermophilic *Methanosaeta ("Methanothrix")* strains as described by Nozhevnikova et al. (17, 18) and Zinder et al. (30, 31) were predominant, and no methanogen of the *Methanosarcina* type was observed in the culture. For further enrichment, the culture was serially diluted in acetate medium containing 100 µg of vancomycin per ml and 1 µg of neomycin per ml.
Although the addition of neomycin resulted in considerable delays of growth, an almost pure culture was obtained from the $10^{-3}$ and $10^{-4}$ dilutions, and thus this procedure was repeated several times. The culture which we obtained contained no contaminants as determined by microscopy. The purity of the culture was also examined by inoculating samples into the following media (no contaminants grew on these media): (i) thioglycolate medium (Difco); (ii) thioglycolate medium containing 10 mM glucose; (iii) thioglycolate medium containing 10 mM cellobiose; (iv) thioglycolate medium containing 10 mM sucrose; (v) thioglycolate medium containing 10 mM xylene; (vi) thioglycolate medium containing 10 mM lactate and 10 mM sulfate; (vii) thioglycolate medium containing 10 mM acetate and 10 mM sulfate; (viii) AC medium (Difco); (ix) acetate medium (Difco); (x) acetate medium containing 10 mM lactate and 10 mM sulfate; (xi) the $H_2-CO_2$ medium used for culture of Methanobacterium thermoautotrophicum (see Materials and Methods). The pure culture was designated strain $P_T$ and was used for further experimentation. The stock culture was routinely maintained in acetate medium without antibiotics.

**FIG. 1.** Photomicrographs of strain $P_T$. (a) Phase-contrast photomicrograph of strain $P_T$. Bar = 10 μm. (b) Phase-contrast photomicrograph of strain $P_T$ after centrifugation at 3,000 × g for 10 min. Bar = 10 μm. (c) Epifluorescence photomicrograph of strain $P_T$. Bar = 10 μm. (d) Colonies of strain $P_T$ in deep agar medium. Bar = 0.5 mm.

**Morphological characteristics of strain $P_T$ and content of coenzyme F₄₂₀ analogs.** Strain $P_T$ cells were nonmotile, straight, sheathed, gram-negative rods with flat ends (Fig. 1a and b). The mean dimensions of single cells were 0.8 by 3.0 μm, and the cells were connected to each other forming characteristic filaments which sometimes were more than 100 μm long. While the filaments sedimentsed during cultivation, they were dispersed homogeneously after gentle shaking. Phase-light particles, which were readily eliminated by centrifugation or sonication (Fig. 1b), were observed in filaments. These particles were very similar to the gas vesicles of thermophilic Methanosaeta strains described by Nozhevnikova and Chudina (17) and Zinder et al. (30).

Our isolate grew on media containing 0 to 0.2 M NaCl. Cells harvested in the late logarithmic phase were not lysed by sodium dodecyl sulfate (0.1g/liter) when we used the procedure described by Boone and Whitman (4). Susceptibility to lysis under hypotonic conditions (4) was not observed.

During isolation, visible colonies developed in deep agar tubes containing acetate medium in dilutions lower than $10^{-4}$. The colonies were white to slightly yellowish and star
TABLE 1. Contents of coenzyme F420 types in methanogens

<table>
<thead>
<tr>
<th>Methanogen</th>
<th>Coenzyme F420 content (mmol/g [dry wt] of cells)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methanosaeta sp. strain PT</td>
<td>63.5 1.9 4.9 43.8 12.9</td>
</tr>
<tr>
<td>Methanosaeta concilii DSM2139</td>
<td>11 0.7 1.4 1.4 7.5</td>
</tr>
<tr>
<td>Methanosaeta concilii DSM3671</td>
<td>6.1 0.4 0.5 0.6 4.6</td>
</tr>
<tr>
<td>Methanosaeta concilii U_A</td>
<td>4.4 0.2 0.9 0.4 2.9</td>
</tr>
<tr>
<td>Methanosaeta concilii PM</td>
<td>3.2 ND 0.3 0.3 2.6</td>
</tr>
<tr>
<td>Methanosarcina barkeri DSM800</td>
<td>258 6.9 4.0 61.1 186</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum DSM1053</td>
<td>697 693 3.3 0.8 ND</td>
</tr>
<tr>
<td>Methanobacterium thermoautotrophicum DSM2133</td>
<td>210 207 1.9 1.5 ND</td>
</tr>
</tbody>
</table>

* ND, Not detected. Glu-2, Glu-3, Glu-4, and Glu-5 indicate the number of glutamyl residues on the side chain of coenzyme F420.

methyl production was observed at pH 6.1 to 7.5 (data not shown). The optimum temperature for methanogenesis was 55°C (Fig. 3). Neither methanogenesis nor growth occurred at 30 or 70°C after 3 weeks of incubation.

Acetate medium was supplemented with organic compounds or hydrogen (H2–CO2, 80/20), and the effects of these additives on methane production were investigated (Table 2). One of these additives, the supernatant of the sludge from which strain PT was isolated, slightly increased methanogenesis, giving a μCH4 of 0.466 day−1. The calculated doubling time was about 1.49 days (36 h). The addition of Polypepton or coenzyme M did not affect methanogenesis, and the addition of 0.1% yeast extract had an inhibitory effect.

**Effects of antibiotics.** The effects of antibiotics on methane formation are shown in Table 3. Inhibition was caused by kanamycin, tetracycline, cycloserine, neomycin, bacitracin, and chloramphenicol at the concentrations tested. Neomycin at a concentration of 1 μg/ml did not have a lethal effect but caused a significant lag period during enrichment (see above).

**Antigenic fingerprint.** Strain PT did not react with any of 18 heterologous S-probes which we used (see Materials and Methods), including the antibodies to Methanosaeta concilii.
Opfikon and Methanosaeta sp. strain CALS-1, as determined by indirect immunofluorescence and the quantitative slide immunoenzymatic assay. A comparative analysis of the antigenic fingerprint of strain P<sub>T</sub> with the fingerprints of reference organisms indicated that strain P<sub>T</sub> was not related antigenically to the well-known methanogens.

**DNA base composition.** The guanine plus cytosine composition of strain P<sub>T</sub> was calculated to be 52.7 mol%. For comparison, DNAs were also extracted from two Methanosaeta strains by the same procedure and analyzed; the guanine-plus-cytosine contents were calculated to be 51.9 and 50.3 mol% for Methanosaeta concilii DSM2139 and DSM3671, respectively.

**DISCUSSION**

On the basis of morphological characteristics, strain P<sub>T</sub> was classified as an acetoclastic methanogen that probably belongs to the genus Methanosaeta (20). Strain P<sub>T</sub> formed a sheathed structure in which many cells were arranged. It could utilize only acetate as a carbon and energy source. Sludge supernatant slightly increased growth and methanogenesis, while yeast extract, peptone, and coenzyme M did not. These characteristics were very similar to those of all other Methanosaeta strains described previously (8, 11, 17, 20, 30). We determined the DNA base composition of strain P<sub>T</sub> to estimate its chemotaxonomic location. The DNA base compositions of strains P<sub>T</sub> and Methanosaeta concilii Opfikon (= DSM2139) and GP6 (= DSM3671) were 52.7, 51.9 and 50.3 mol%, respectively. The values for strains Opfikon and GP6 were recently determined by Touzel et al. (26) using the thermal denaturation procedure to be 52.3 and 50.2 mol%. The values calculated by us for these strains were in good agreement with the values reported by Touzel et al. Recently, Zinder determined the DNA base composition of his thermophilic strain CALS-1 to be 50 mol% (29). These results indicate that strain P<sub>T</sub> should be assigned to the genus Methanosaeta.

Strain P<sub>T</sub> contained phase-light particles that were apparently similar to the gas vesicles of thermophilic Methanosaeta strains (17, 30). Zinder et al. have suggested that gas vesicles are common in thermophilic Methanosaeta strains (30). However, on rare occasions we have observed phase-light particles even in Methanosaeta strains in mesophilic anaerobic digesters (unpublished data). Thus, the formation of gas vesicles may depend on environmental or physiological conditions rather than on thermophilic growth.

In contrast to the finding that long filaments are rare in Methanosaeta sp. strain CALS-1 (30), our isolate formed filaments that were sometimes more than 100 μm long. Long filaments were also observed when strain P<sub>T</sub> was cultivated in CALS-1 medium (data not shown), indicating that the difference in the contents of the two media was not responsible for the formation of long filaments.

One of the most characteristic features of strain P<sub>T</sub> was that it autofluoresced, as determined by epifluorescence microscopy. In general, Methanosaeta strains, including the strains of thermophilic species, exhibit very weak or no autofluorescence because of the low level of coenzyme F<sub>420</sub> in the cells (10, 28). Quantification of coenzyme F<sub>420</sub> in the strain P<sub>T</sub> cells by HPLC revealed that they contained a higher level of coenzyme F<sub>420</sub> than the cells of other strains. The predominant component was coenzyme F<sub>20</sub> with four glutamyl residues rather than coenzyme F<sub>420</sub> with five glutamyl residues, which was the predominant coenzyme F<sub>420</sub> type in other Methanosaeta strains (Table 1).

A comparative analysis of the antigenic fingerprint of strain P<sub>T</sub> with the fingerprints of reference methanogens, which is a very useful way to distinguish organisms at the species level, also showed the novelty of our isolate. Strain P<sub>T</sub> was not antigenically related to the well-known methanogens, including the closely related organisms Methanosaeta sp. strain CALS-1 and Methanosaeta concilii Opfikon, suggesting that this isolate is a new, as yet undescribed methanogen.

**TABLE 3.** Effect of antibiotics on methanogenesis by strain P<sub>T</sub>.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Conc (μg/ml)</th>
<th>Relative formation of methane (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Penicillin G</td>
<td>500</td>
<td>96</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>100</td>
<td>93</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>100</td>
<td>95</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>Cycloserine</td>
<td>10</td>
<td>34</td>
</tr>
<tr>
<td>Neomycin</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>Bacitracin</td>
<td>10</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>5</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

The amount of methane formed was determined after 10 days of cultivation at 55°C. The amount of methane formed in the control culture (acetate medium without antibiotic) was defined as 100%.

**ACKNOWLEDGMENTS**

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**REFERENCES**

29. Zinder, S. H. Personal communication.