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 P1, 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 P1 with the fingerprints of reference organisms showed that this isolate was not related antigenically to the reference methanogens, including Methanosaeta sp. ("Methanothrix" sp.) strain CALS-1 and Methanosaeta concilii ("Methanothrix soehngenii") Opfikon. Strain P1 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 P1 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 per day 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 thermosautrophicum DSM1053 and DSM2133, Methanosarcina barkeri DSM800, and Methanosaeta concilii DSM2139 ("Methanothrix soehngenii" Opfikon) and DSM3671 (= GP6) were obtained from the Deutsche Sammlung von Mikroorganismen, Braunschweig, Federal Republic of Germany. Methanosaeta concilii U_A and P_M 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). Slightly 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 N_2 and CO_2 (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 119 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 120 of the Deutsche Sammlung von Mikroorganismen (5).

*Methanosaeta concilii* DSM2139 ("Methanothrix soehngenii") Opfikon, DSM3671, Uα, and Pα were cultivated at 37°C under the same conditions as strain Pα.

**Microscopy.** An exponential-phase culture was observed by phase-contrast microscopy and epifluorescence microscopy, using an Olympus model BHS-RFK microscope. 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.

**Analytical methods.** The amount of methane produced was determined by gas chromatography by using a molecular sieve column connected to a Shimadzu model GC-8AIT thermal conductivity detector. Argon was used as the carrier gas. The amount of acetate was determined by gas chromatography by using a Shimadzu model LC-6A system. The guanine-plus-cytosine content was determined by reversed-phase HPLC (Shimadzu model LC-6A system) supplemented with fluorometric detection; our system was based on the system described by van Boeckel et al. (27) and Gorris et al. (9, 10).

**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 xylose; (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; (x) acetate medium containing 10 mM sulfate; (x) acetate medium containing 10 mM lactate; and (xi) the $\text{H}_2\text{CO}_3$ 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.

Morphological characteristics of strain $P_T$ and content of coenzyme $F_{420}$ 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 $\mu$m, and the cells were connected to each other forming characteristic filaments which sometimes were more than 100 $\mu$m long. While the filaments sedimented 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 Methanoseta 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 (nmol/g [dry wt] of cells)²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total  Glu-2  Glu-3  Glu-4  Glu-5</td>
</tr>
<tr>
<td>Methanosaeta sp. strain P₅</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 U₅</td>
<td>4.4     0.2     0.9     0.4     2.9</td>
</tr>
<tr>
<td>Methanosaeta concilii P₅</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 thermautotrophicum DSM1053</td>
<td>697  693  3.3     0.8     ND</td>
</tr>
<tr>
<td>Methanobacterium thermautotrophicum DSM2133</td>
<td>210  207  1.9     1.5     ND</td>
</tr>
</tbody>
</table>

¹ 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.

methane 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 P₅ 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 P₅ 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 Pₜ with the fingerprints of reference organisms indicated that strain Pₜ was not related antigenically to the well-known methanogens.  

**DNA base composition.** The guanine plus cytosine composition of strain Pₜ 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 morphoclastic characteristics, strain Pₜ was classified as an acetoclastic methanogen that probably belongs to the genus Methanosaeta (20). Strain Pₜ 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 Pₜ to estimate its chemotaxonomic location. The DNA base compositions of strains Pₜ 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 Pₜ should be assigned to the genus Methanosaeta.

Strain Pₜ 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 digestors (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 Pₜ 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 Pₜ was that it autofluoresced, as determined by epifluorescence microscopy. In general, Methanosaeta strains, including the species of thermophilic species, exhibit very weak or no autofluorescence because of the low level of coenzyme F₄₂₀ in the cells (10, 28). Quantification of coenzyme F₄₂₀ in the strain Pₜ cells by HPLC revealed that they contained a higher level of coenzyme F₄₂₀ than the cells of other strains. The predominant component was coenzyme F₄₂₀ with four glutamyl residues rather than coenzyme F₄₂₀ with five glutamyl residues, which was the predominant coenzyme F₄₂₀ type in other Methanosaeta strains (Table 1).

A comparative analysis of the antigenic fingerprint of strain Pₜ 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 Pₜ 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 immunotype. On the basis of the findings described above, we believe that strain Pₜ is a novel Methanosaeta strain. It should be useful not only in thermophilic anaerobic digestors to enhance the activity in the digestors, in which the conversion of acetate to methane is slow, but also in biochemical analysis, particularly in studies of the function of coenzyme F₄₂₀ in acetoclastic methanogenesis.

*Methanosaeta* sp. strain Pₜ has been deposited in the culture collection of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany (DSM 6194).

### ACKNOWLEDGMENTS

We thank Everly Conway de Macario and Alberto J. L. Macario for the antigenic fingerprinting of our isolate. We also thank Kazuhiro Tanaka of our laboratory for much technical advice concerning anaerobic cultivation.

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### REFERENCES


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**TABLE 2. Effect of additives on μₘₜₜ for strain Pₜ**

<table>
<thead>
<tr>
<th>Additive</th>
<th>μₘₜₜ (day⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.409 ± 0.006</td>
</tr>
<tr>
<td>H₂CO₃</td>
<td>0.377 ± 0.003</td>
</tr>
<tr>
<td>Polypepton (0.1%)</td>
<td>0.421 ± 0.001</td>
</tr>
<tr>
<td>Yeast extract (0.1%)</td>
<td>0.399 ± 0.017</td>
</tr>
<tr>
<td>Coenzyme M (2 mM)</td>
<td>0.412 ± 0.007</td>
</tr>
<tr>
<td>Sludge supernatant (3%)</td>
<td>0.466 ± 0.003</td>
</tr>
<tr>
<td>Rumen fluid (8%)</td>
<td>0.314 ± 0.033</td>
</tr>
</tbody>
</table>

* Duplicate cultures were incubated at 55°C.

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**TABLE 3. Effect of antibiotics on methanogenesis by strain Pₜ**

<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%.


