Isolation and Characterization of New Methanothrix Strains

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Mesophilic Methanothrix sp. strains MTAS and MTKO were isolated as pure cultures from the sludge of anaerobic digesters. We examined the relationships between these two isolates and three other Methanothrix strains (strains Opfikon\(^T\) \(T = \) type strain), GP6\(^T\), and FE) by using an immunological method, by performing DNA homology experiments, and by analyzing polar lipids. Our results indicate that all five strains belong to the same species.

Acetate is the precursor of at least 60% of the methane produced during anaerobic digestion of organic compounds (4, 20). Kaspar and Wührmann (13) reported that acetate splitting is the rate-limiting reaction in this system. On the other hand, the members of three genera, Methanothrix, Methanoseta, and Methanosarcina, can convert acetate to methane. The members of the genus Methanothrix, in particular, have a high affinity for acetate \((K_a, 0.7 \text{ mM})\), so these bacteria seem to play an important role in methane fermentation (8, 12).

The genus Methanothrix includes two mesophilic species, Methanothrix soehngenii (type strain, Opfikon) (12) and Methanothrix concilii (type strain, GP6) (17). Touzel et al. indicated that strains Opfikon\(^T\) \(T = \) type strain) and GP6\(^T\) belong to the same species, as judged by the results of DNA-DNA hybridization experiments, and proposed that the name Methanothrix concilii should be rejected as a later synonym of Methanothrix soehngenii (22). Recently, Patel and Sprott proposed that the genus name Methanothrix should be rejected and described a new genus, Methanoseta, with Methanoseta concilii as its type species, because no axenic culture of Methanothrix soehngenii seems to exist (18). In this paper we describe the isolation of two new Methanothrix strains as pure cultures (strains MTAS and MTKO), and we compare these strains with other Methanothrix strains.

MATERIALS AND METHODS

Sources of strains. Methanothrix concilii GP6\(^T\) (= OCM69\(^T\)) was obtained from the Oregon Collection of Methanogens, Beaverton. Methanothrix soehngenii Opfikon \(^T\) (= DSM 2139\(^T\)) was a gift from A. J. B. Zehnder (Agricultural University, Wageningen, The Netherlands). Methanobacterium thermoautotrophicum \(\Delta H^T\) (= DSM 1053\(^T\)), Methanosarcina barkeri MS\(^T\) (= DSM 800\(^T\)), and Methanothrix soehngenii FE (= DSM 3013) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Federal Republic of Germany. Methanothrix sp. strain MTAS was isolated from the anaerobic digestor (in which the temperature is not controlled) that is used for treating domestic wastewater in the town of Ashiya (Fukuoka, Japan). Strain MTKO was isolated from the anaerobic fixed-bed digestor (in which the temperature is not controlled) that is used for treating wastewater from a flush toilet in our laboratory.

Media and cultivation. We used Deutsche Sammlung von Mikroorganismen und Zellkulturen medium no. 334 (7) for isolating and cultivating all Methanothrix strains except strain GP6\(^T\). This medium was dispensed into serum vials under 100% nitrogen atmosphere and autoclaved at 121°C for 20 min. The sterile medium was reduced with 1.7 \(\mu\)M cysteine and 1.2 \(\mu\)M Na\(\_\)S before it was used. The pH was adjusted to 7.0 by adding 1 M HCl.

Methanothrix concilii GP6\(^T\) (17) and Methanobacterium thermoautotrophicum \(\Delta H^T\) (16) were cultivated as described previously. Methanosarcina barkeri MS\(^T\) was cultivated in BCYT medium that contained 10 ml of methanol per liter (21).

Enrichment and isolation. Enrichment was performed in 1,000-ml screw-cap laboratory bottles. Aliquots (250 ml) of inoculum from each anaerobic digestor were mixed with 250-ml portions of medium. Cultures were incubated at 37°C under a nitrogen atmosphere without shaking. At 1-week intervals, inoculum from each laboratory bottle was transferred to fresh medium (50%, vol/vol). After 3 months, each enriched culture was serially diluted in medium that contained 0.1 mg of vancomycin per ml, and the preparations were incubated at 37°C. The cultures at the highest dilution at which bacterial growth was detected were checked for purity. When contaminants were detected, the serial dilution steps were repeated. Pure cultures were stored at \(-80°C\) in growth medium that contained glycerol (15%, vol/vol).

Assessment of purity. The purity of cultures was assessed by inoculating samples (5%, vol/vol) into the following media: thioglycolate medium (WAKO Pure Chemical Industries, Ltd., Osaka, Japan); PCM-1 medium, a growth medium that contains yeast extract (0.5%, wt/vol), Bactopeptone (2%, wt/vol), glucose (0.25%, wt/vol), sucrose (0.25%, wt/vol), cellobiose (0.25%, wt/vol), and xylose (0.25%, wt/vol) instead of sodium acetate; and PCM-2 medium, a growth medium that contains 10 mM sodium lactate and 10 mM sodium sulfate instead of sodium acetate. Inoculated media were incubated for 45 days at 37°C. Cultures were examined under a light microscope.

pH and temperature dependence. To examine the effects of pH and temperature on growth, triplicate experiments were performed in 120-ml serum vials containing 50 ml of bicarbonate-free medium supplemented with 4 mM acetate. The pH was controlled with 50 mM MES [2-(N-morpholino)ethanesulfonic acid] buffer or 50 mM HEPEs (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer at the temperature of incubation. The pH and temperature were 7.0 and 37°C, respectively, unless otherwise indicated.

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Analytical techniques. Methane production was determined with a gas chromatograph (model GC-9A; Shimadzu Corp., Kyoto, Japan) equipped with a flame ionization detector and a column (3 m by 3 mm [inside diameter]) packed with Porapak T (50-60 mesh; Waters Associates, Inc., Milford, Mass.). The column temperature was 80°C, and the carrier gas was nitrogen (60 ml/min).

A specific rate constant for methane production (μCH4) was calculated from the slope of the logarithm of the methane content as a function of time. Slopes were calculated by using least-squares regression analysis.

Immunological relationships. Antisera against Methanotrix strains MTAS, MTKO, and FE were raised in rabbits as previously described (5). Booster doses were administered three times at intervals of 3 weeks because of the low antigenicity of Methanotrix spp. (14). Cross-reactivity was measured with an S probe (described by Conway de Macario et al. [5]) by using the indirect immunofluorescence technique (5, 6).

Preparation of DNA. Cells were harvested at late exponential phase from 5-liter cultures. The harvested cells were washed with a solution containing 0.15 M NaCl and 0.1 M EDTA (pH 8.0) and were stored at −20°C prior to use.

Cells were lysed by using the procedure described by Patel and Spott (18). After incubation with RNase A (Boehringer Mannheim, Federal Republic of Germany) and then with proteinase K (Boehringer GmbH), the protein was removed by using chloroform-isooamyl alcohol (24:1, vol/vol). The crude DNA was concentrated with an Ultrafree-20 filter unit (Millipore Corp., Bedford, Mass.) and purified by high-pressure liquid chromatography (HPLC).

HPLC was performed by using a system consisting of a Tosoh CCPM pump and a model SC-8010 system controller (Tosoh Corp., Tokyo, Japan), with detection at 260 nm.

A column of hydroxyapatite (Bio-Gel HPHT; Bio-Rad Laboratories, Tokyo, Japan), with detection at 260 nm. The physiological characteristics of strains MTAS and MTKO. (i) Isolation and morphology. After two sets of serial dilutions, all contaminants were eliminated, and pure cultures of strains MTAS and MTKO were obtained. The active cultures have been maintained for 2 years by subculturing them at intervals of 3 or 4 weeks in antibiotic-free medium. The purity of these cultures has been assessed every 6 months, and no contaminants have been detected.

Isolation of members of the genus Methanotrix is difficult because of the inability of the organisms to grow on solid media and the presence of a sheath which could be involved in the adhesion of other bacteria to filaments (22). However, we were able to isolate two Methanotrix strains as pure cultures by using only the serial dilution method. The sources of our isolates were collected from anaerobic digestors in which the temperature is not controlled, and the temperatures in these digestors are certainly lower than the temperatures in mesophilic anaerobic digestors. Therefore, the reason for our success may be that the bacterial floras of the sources of our isolates were different than the floras of the mesophilic digestors which were the sources of Methanotrix strains described previously (12, 22), so that the numbers of bacteria that adhered strongly to the sheaths of the Methanotrix cells were small.

The cells of our isolates were rod shaped (about 1.0 by 2.0 μm) and formed long filaments in standing cultures. Both of our strains were gram negative.

(ii) Physiology. The physiological characteristics of strains MTAS and MTKO were similar. Cells grew on acetate as a sole source of carbon and energy, but they were not able to utilize H2-CO3, methanol, methylamine, and formate. Growth occurred over a pH range from 6.0 to 8.0, and the optimum pH was between 6.5 and 7.5. No growth was observed at pH 5.5 or 8.5 (Fig. 1). The optimum pH ranges
FIG. 1. Dependence on pH of production of methane from acetate by Methanothrix strains MTAS and MTKO. The data are averages of the results of three experiments. Bars indicate standard deviations. Symbols: ○, strain MTAS in medium buffered with 50 mM MES; ■, strain MTKO in medium buffered with 50 mM HEPES; ◯, strain MTKO in medium buffered with 50 mM MES; ●, strain MTAS in medium buffered with 50 mM HEPES.

for growth were pH 7.4 to 7.8 for strain Opfikon \(^7\) (12) and pH 7.1 to 7.5 for strain GP6 \(^7\) (17). Also, strain Opfikon \(^7\) did not grow at pH 6.5 (12), and the methane production rate of strain GP6 \(^7\) at pH 6.6 was 55% of the methane production rate at the optimum pH (17). Thus, our isolates had a broad pH range for growth compared with strains Opfikon \(^7\) and GP6 \(^7\).

The optimum temperature for growth was between 37 and 40°C. At 45°C no growth was observed (Fig. 2). The average doubling times of strains MTAS and MTKO were 44.8 and 44.2 h, respectively, under optimal growth conditions.

**Taxonomy.** (i) **Immunological relationships.** The S titers were 1:100 in our experiments. The S probes for our two isolates and strain FE gave +4 reactions with strain GP6 \(^7\) and +3 or +2 reactions with strain Opfikon \(^7\). The cross-reactions with strains MTAS and MTKO were +2 or +3.

The S probes did not react at all with Methanosarcina barkeri MST \(^7\), and the S probes for strains FE and MTAS gave only slight cross-reactions with Methanobacterium thermoautotrophicum ΔH \(^7\); these very weak cross-reactions may have been nonspecific cross-reactions which were the result of having to use concentrated S probes because of the low antigenicity of Methanothrix spp.

The results described above demonstrated that our isolates were immunologically related to other *Methanothrix* strains and especially to strain GP6 \(^7\). Touzel et al. (22) and Macario and Conway de Macario (14) reported that Methanothrix-specific antisera were strain specific. In contrast to the results of these authors, our antisera recognized all strains of *Methanothrix* spp. Therefore, these sera should be useful for detecting and enumerating *Methanothrix* cells in environmental samples.

Our results also suggest that the structure of the cell surface is heterogeneous within the genus *Methanothrix*, as described by Macario and Conway de Macario (14), and that the structure of our isolates resembles the structure of strain GP6 \(^7\).

(ii) **G+C contents and DNA-DNA hybridization.** The G+C contents of the *Methanothrix* strains fell within a limited range (50.83 to 51.31 mol%) (Table 1). Furthermore, the *Methanothrix* strains did not differ from each other on the basis of the results of the DNA-DNA hybridization experiments (levels of homology, 78 to 104%) (Table 1). These results agree with those reported previously (22).

(iii) **Composition of polar lipids.** Figure 3 shows the pattern which was obtained by performing two-dimensional thin-layer chromatography of the total lipids from strain MTAS. The patterns for strains MTKO and FE were very similar to the pattern for strain MTAS. The spots of polar lipids were detected by acid charring and were classified by using appropriate staining tests. The various spots were identified as shown in Fig. 3.

The structures of eight lipids were identified as follows: GL2a, 2,3-di-O-phytanyl-1-O-[a-0-mannopyranosyl-(1→3)-\(\beta\)-D-galactopyranosyl]-sn-glycerol; GL2b, 2-O-phytanyl-3-O-[3'-hydroxy-3',7',11',15'-tetramethylhexadecyl]-1-O-[\(\beta\)-D-galactopyranosyl-(1→6)-\(\beta\)-D-galactopyranosyl]-sn-glycerol; PIL, 2,3-di-O-phytanyl-sn-glycero-1-phosphoryl-1'-myo-

![FIG. 2. Effect of growth temperature on production of methane by *Methanothrix* strains MTAS and MTKO. The data are averages of the results of three experiments. Bars indicate standard deviations. Symbols: ■, strain MTKO; ○, strain MTAS.](image-url)

<table>
<thead>
<tr>
<th>Strain</th>
<th>G+C content (mol%)</th>
<th>% Homology with (^3)H-labeled DNA from:</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Strain GP6(^7)</td>
</tr>
<tr>
<td><em>Methanothrix</em> concili GP6(^7)</td>
<td>50.85 ± 0.17(^a)</td>
<td>100</td>
</tr>
<tr>
<td><em>Methanothrix</em> soehngenii Opfikon(^7)</td>
<td>51.25 ± 0.22</td>
<td>94</td>
</tr>
<tr>
<td><em>Methanothrix</em> soehngenii FE</td>
<td>50.83 ± 0.11</td>
<td>88</td>
</tr>
<tr>
<td><em>Methanothrix</em> sp. strain MTAS</td>
<td>50.83 ± 0.03</td>
<td>86</td>
</tr>
<tr>
<td><em>Methanothrix</em> sp. strain MTKO</td>
<td>51.31 ± 0.21</td>
<td>104</td>
</tr>
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</table>

\(^a\) Mean ± standard deviation (\(n = 3\)).
Inositol; PL, 2,3-di-O-phytanyl-sn-glycerophosphate; PNL1a, 2,3-di-O-phytanyl-sn-glycerol-1-phosphatidylethanolamine; PNL1b, 2-O-phytanyl-3-O-(3'-hydroxy-3',7',11',15'-tetramethyl)hexadecyl-sn-glycerol-1-phosphatidylethanolamine; PNL2a, 2,3-di-O-phytanyl-sn-glycerol-1-phosphatidylserine; and PNL2b, 2-O-phytanyl-3-O-(3'-hydroxy-3',7',11',15'-tetramethyl)hexadecyl-sn-glycerol-1-phosphatidylserine.

From a comparison of our results with the data of Ferrante et al. (9, 10), we assumed that GL1 contained 2,3-di-O-phytanyl-1-O-(β-galactopyranosyl)-sn-glycerol and its 3'-hydroxydeether analog and that GL3 was 2-O-phytanyl-3-O-(3'-hydroxy-3',7',11',15'-tetramethyl)hexadecyl-1-O-(β-D-galactopyranosyl-1→6)-(β-D-glucopyranosyl-1→3)]-β-D-galactopyranosyl-sn-glycerol.

The compositions of the polar lipids are shown in Table 2. Ferrante et al. investigated the polar lipids of strain GP6 in detail (9-11). In this study, we detected all of the major lipids in strain GP6 in the other three strains. Furthermore, we identified three minor lipids (PL, PNL2a, and PNL2b) which were not reported in strain GP6. As shown in Table 2, the levels of the minor lipids were different in the different strains. However, the synthesis of these lipids seems to be easily influenced by growth conditions. Therefore, the polar lipid compositions appear to be very similar for all of the strains.

As mentioned above, our pure isolates exhibited phenotypic and genotypic similarities to other Methanothrix strains. Consequently, the strains which we studied clearly belong to the species described by Touzel et al. (22). This conclusion may contribute to decisions concerning appropriate nomenclature.

Strain MTAS (FERM P-11824) has been deposited in the culture collection of the Fermentation Research Institute, Ibaraki, Japan, as a patented microorganism. We are also planning to deposit strain MTKO in one of the culture collections.

**FIG. 3.** Pattern after two-dimensional thin-layer chromatography of the total lipids from Methanothrix sp. strain MTAS. The solvents were chloroform-methanol-7 M aqueous ammonia (60:35:8, vol/vol/vol) in the vertical direction and chloroform-methanol-acetic acid-water (85:30:15:5, vol/vol/vol) in the horizontal direction.

**TABLE 2.** Polar lipids of Methanothrix strains

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Strain</th>
<th>Strain</th>
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<th>Strain</th>
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<tr>
<td></td>
<td>FE</td>
<td>MTAS</td>
<td>MTKO</td>
<td>GP6</td>
</tr>
<tr>
<td>Glycolipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GL1</td>
<td>8.5</td>
<td>15.9</td>
<td>12.8</td>
<td>2.4</td>
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<tr>
<td>GL2a</td>
<td>22.5</td>
<td>20.0</td>
<td>21.6</td>
<td>37.8</td>
</tr>
<tr>
<td>GL2b</td>
<td>13.0</td>
<td>17.0</td>
<td>19.1</td>
<td>19.2</td>
</tr>
<tr>
<td>GL3</td>
<td>4.4</td>
<td>2.5</td>
<td>3.5</td>
<td>0.2</td>
</tr>
<tr>
<td>GL4</td>
<td>4.6</td>
<td>3.0</td>
<td>3.4</td>
<td>ND</td>
</tr>
<tr>
<td>Phospholipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PIL</td>
<td>33.5</td>
<td>25.9</td>
<td>26.8</td>
<td>25.6</td>
</tr>
<tr>
<td>PL</td>
<td>3.7</td>
<td>2.2</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Aminophospholipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PNL1a</td>
<td>2.9</td>
<td>2.8</td>
<td>3.4</td>
<td>4.2</td>
</tr>
<tr>
<td>PNL1b</td>
<td>7.9</td>
<td>7.0</td>
<td>5.7</td>
<td>10.3</td>
</tr>
<tr>
<td>PNL2a</td>
<td>Tr</td>
<td>2.0</td>
<td>1.8</td>
<td>ND</td>
</tr>
<tr>
<td>PNL2b</td>
<td>Tr</td>
<td>1.8</td>
<td>1.6</td>
<td>ND</td>
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</tbody>
</table>

*The designations for the polar lipids are the same as those used in Fig. 3.*

*The values for strain GP6 were recalcualted from the data of Ferrante et al. (9, 10).*

*ND, not detected.

*Tr, trace.*

**ACKNOWLEDGMENTS**

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