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 digestors. We examined the relationships between these two isolates and three other *Methanothrix* strains (strains Opfikon*\textsuperscript{T} [T = type strain], GP6*, 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 ($K_s$, 0.7 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*\textsuperscript{T} and GP6* 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 Sprock proposed that the genus name *Methanothrix* should be upheld and described a new genus, *Methanaseta*, 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* ( = OCM69*) was obtained from the Oregon Collection of Methanogens, Beaverton. *Methanothrix soehngenii* Opfikon* ( = DSM 2139*) was a gift from A. J. B. Zehnder (Agricultural University, Wageningen, The Netherlands). *Methanobacterium thermoautotrophicum* ΔH* ( = DSM 1053*), *Methanosarcina barkeri* MS* ( = DSM 800*), 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.

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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* . 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 μM cysteine and 1.2 μM Na$_2$S before it was used. The pH was adjusted to 7.0 by adding 1 M HCl.

*Methanothrix concilii* GP6* (17) and *Methanobacterium thermoautotrophicum* ΔH* (16) were cultivated as described previously. *Methanosarcina barkeri* MS* 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), Bacto- Peptone (2%, wt/vol), glucose (0.25%, wt/vol), succrose (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.
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 ($\mu_{CH_4}$) 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 Methanothrix 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 Methanothrix 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 Dyer (18). After incubation with RNase A (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 HPPHT; Bio-Rad Laboratories, Richmond, Calif.) was used for separation. The flow rate was 0.4 ml/min. The initial solvent was 100 mM phosphate buffer (pH 6.8) containing 0.01 mM CaCl$_2$. At 15 min after injection, a linear gradient program (100 to 400 mM phosphate buffer [pH 6.8], containing 0.01 mM CaCl$_2$ for 30 min) was started. The fractions containing DNA were collected with a model FRAC-100 fraction collector (Pharmacia, Uppsala, Sweden).

G+C content of DNA. The G+C content of DNA was measured by HPLC (15). A 50-µl aliquot of a 1× SSC solution (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 10 µg of DNA was heated at 100°C for 15 min and cooled rapidly in an ice bath. Then 10 µl of a nuclease P1 solution (5 U of nuclease P1 per ml in 40 mM sodium acetate buffer containing 0.2 mM ZnCl$_2$ [pH 5.3]; Yamasa Shoyu Co., Ltd., Choshi, Japan) was added, and the mixture was incubated at 50°C for 90 min. After this, 10 µl of a 1 M glycine solution containing 10 mM MgCl$_2$ and 10 mM ZnCl$_2$ (pH 9.0) and 10 µl of a solution containing alkaline phosphatase from bovine intestinal mucosa (50 U/ml in 0.5 M Tris buffer [pH 8.5]; Sigma Chemical Co., St. Louis, Mo.) were added. The resulting mixture was incubated at 37°C for 6 h. The nucleosides produced were quantified by HPLC.

We used the GC Analysis Standard (Yamasa Shoyu Co., Ltd.) as the standard of quantification; this product contains equimolar amounts of the sodium salts of dCMP, dAMP, dGMP, and dTMP, and we prepared an equimolar mixture of nucleosides from this product by using alkaline phosphatase, as described above.

HPLC was performed by using a model LC-6A apparatus (Shimadzu Corp.). The conditions were as follows: column, STR ODS-H (4 mm [inside diameter] by 150 mm; Shimadzu Techno-Research, Inc., Kyoto, Japan); temperature, 40°C; mobile phase, 0.2 M NH$_4$H$_2$PO$_4$-acetanitride (40:1, vol/vol); flow rate, 1.0 ml/min; detector, UV radiation at 270 nm.

DNA-DNA hybridization. Hybridization was performed by using the membrane filter method (1). 3H-labeled DNAs were prepared by using a nick translation kit (Takara Shuzo Co., Ltd., Kyoto, Japan). Unlabeled single-stranded DNAs were immobilized on nitrocellulose filters (type TM-3; pore size, 0.3 µm; diameter, 13.0 mm; Toyoo Roshi Kaisha, Ltd., Tokyo, Japan). We mixed labeled single-stranded DNA (1%, wt/wt) with unlabeled single-stranded DNA and incubated the mixture at 67°C for 40 h. Reassociation experiments were performed in triplicate, and the resulting data were averaged.

Composition of polar lipids. Cells were harvested aerobically at the late exponential phase and were washed with distilled water by using a Minint system equipped with 0.2-µm-pore-size membrane filters (Millipore Corp.). Washed cells were stored at −80°C prior to extraction of lipids.

After disruption of cells by passage through a French pressure cell (SLM Instruments, Inc., Urbana, Ill.) at 138 MPa, lipids were extracted by using the method of Bligh and Dyer (3). Lipid species were separated and purified by two-dimensional thin-layer chromatography (16). Glycolipids and phospholipids were determined by using the methods described by Roughan and Batt (19) and Bartlett (2), respectively.

RESULTS AND DISCUSSION

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 Methanothrix 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 adherence of other bacteria to filaments (22). However, we were able to isolate two Methanothrix 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 flora of the sources of our isolates were different than the flora of the mesophilic digestors which were the sources of our isolates were different than the floras of the mesophilic digestors. Therefore, the reason for our success may be that the bacterial flora of the sources of our isolates were different than the floras of the mesophilic digestors (12, 22), so that the numbers of bacteria that adhered strongly to the sheaths of the Methanothrix cells were small.

The cells of our isolates were rod shaped (about 1.0 by 2.0 pm) 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 H$_2$, CO$_2$, 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...
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\(^\circ\)C. At 45\(^\circ\)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 titters 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* MT \(^7\), and the S probes for strains FE and MTAS gave only slight cross-reactions with *Methanobacterium thermoautotrophicum* \(\Delta H^\circ\); 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 had a broad basis of the results of the DNA-DNA hybridization experiments. 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-D-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-glycerol-1-phosphoryl-1′-myo-

**TABLE 1. Levels of DNA homology among *Methanothrix* strains**

<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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<tbody>
<tr>
<td></td>
<td></td>
<td>Strain GP6 (^7)</td>
</tr>
<tr>
<td><em>Methanothrix concilii</em> GP6 (^7)</td>
<td>50.85 ± 0.17(^a)</td>
<td>100</td>
</tr>
<tr>
<td><em>Methanothrix soehngenii</em> Opfikon (^7)</td>
<td>51.25 ± 0.22</td>
<td>94</td>
</tr>
<tr>
<td><em>Methanothrix soehngenii</em> FE</td>
<td>50.83 ± 0.11</td>
<td>88</td>
</tr>
<tr>
<td><em>Methanothrix sp.</em> strain MTAS</td>
<td>50.83 ± 0.03</td>
<td>86</td>
</tr>
<tr>
<td><em>Methanothrix sp.</em> strain MTKO</td>
<td>51.31 ± 0.21</td>
<td>104</td>
</tr>
</tbody>
</table>

\(^a\) Mean ± standard deviation \((n = 3)\).
belong to the species described by Touzel et al. (22). This was not reported in strain GP6T. Lipid compositions appear to be very similar for all of the strains. However, the synthesis of these lipids seems to be easily influenced by growth conditions. Therefore, the polar lipids of strain GP6T in this study, we detected all of the major lipids identified three minor lipids (PL, PNL2a, and PNL2b) which were not reported in strain GP6T.

From a comparison of our results with the data of Ferrante et al. (9,10), the compositions of the polar lipids are shown in Table 2. Ferrante et al. investigated the polar lipids of strain GP6T in detail (9-11). In this study, we detected all of the major lipids and their 3'-hydroxydiether analog and that GL3 was 2-0-phytanyl-3-0-(3'-hydroxy-3',7',11',15'-tetramethyl)hexadecyl-1-O-(beta-D-galactopyranosyl)sn-glycero-1-phosphatidylethanolamine.

The compositions of the polar lipids are shown in Table 2. The values for strain GP6T were recalculated from the data of Ferrante et al. (9,10). The designation for the polar lipids are the same as those used in Fig. 3. The values for strain GP6T were not detected. Tr, trace.

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**REFERENCES**


