Methanolinea mesophila sp. nov., a hydrogenotrophic methanogen isolated from rice field soil, and proposal of the archaeal family Methanoregulaceae fam. nov. within the order Methanomicrobiales

Sanae Sakai,1 Masayuki Ehara,1,2 I-Cheng Tseng,3 Takashi Yamaguchi,2 Suzanna L. Bräuer,4 Hinsby Cadillo-Quiroz,5 Stephen H. Zinder6 and Hiroyuki Imachi1

Correspondence
Sanae Sakai
sakai-s@jamstec.go.jp

1Subsurface Geobiology Advanced Research (SUGAR) Project, Extremobiosphere Research Program, Institute of Biogeosciences, Japan Agency for Marine-Earth Science & Technology (JAMSTEC), Yokosuka, Kanagawa 237-0061, Japan
2Department of Environmental Systems Engineering, Nagaoka University of Technology, Nagaoka, Niigata 940-2188, Japan
3Department of Biology, National Cheng Kung University, Tainan 701, Taiwan, ROC
4Department of Biology, Appalachian State University, Boone, NC 28608, USA
5School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA
6Department of Microbiology, Cornell University, Ithaca, NY 14853, USA

A novel mesophilic, hydrogenotrophic methanogen, designated strain TNR1T, was isolated from an anaerobic, propionate-degradation enrichment culture that was originally established from a rice field soil sample from Taiwan. Cells were non-motile rods, 2.0–6.5 μm long by 0.3 μm wide. Filamentous (up to about 100 μm) and coccoid (about 1 μm in diameter) cells were also observed in cultures in the late exponential phase of growth. Strain TNR1T grew at 20–40 °C (optimally at 37 °C), at pH 6.5–7.4 (optimally at pH 7.0) and in the presence of 0–25 g NaCl l−1 (optimally at 0 g NaCl l−1). The strain utilized H2/CO2 and formate for growth and produced methane. The G + C content of the genomic DNA was 56.4 mol%. Based on sequences of both the 16S rRNA gene and the methanogen-specific marker gene mcrA, strain TNR1T was related most closely to Methanolinea tarda NOBI-1T; levels of sequence similarities were 94.8 and 86.4 %, respectively. The 16S rRNA gene sequence similarity indicates that strain TNR1T and M. tarda NOBI-1T represent different species within the same genus. This is supported by shared phenotypic properties, including substrate usage and cell morphology, and differences in growth temperature. Based on these genetic and phenotypic properties, strain TNR1T is considered to represent a novel species of the genus Methanolinea, for which the name Methanolinea mesophila sp. nov. is proposed; the type strain is TNR1T (=NBRC 105659T =DSM 23604T). In addition, we also suggest family status for the E1/E2 group within the order Methanomicrobiales, for which the name Methanoregulaceae fam. nov. is proposed; the type genus of family is Methanoregula.

The genus Methanolinea, belonging to the order Methanomicrobiales, was described as an H2/CO2-using methanogenic archaeon (Imachi et al., 2008). The genus currently consists of only one species, Methanolinea tarda, the type strain of which, NOBI-1T, was isolated from a methanogenic sludge treating municipal sewage sludge (Imachi et al., 2008). Moreover, several 16S rRNA gene surveys have retrieved Methanolinea-related clones from a variety of anaerobic environments, including methanogenic sludge (Chen et al., 2004, 2009; Diaz et al., 2006; Imachi et al., 2008; Lykidis et al., 2011; Narihiro et al., 2009; Sakai et al., 2009; Yashiro et al., 2011), marine

Abbreviation: FISH, fluorescence in situ hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and mcrA gene sequences of strain TNR1T are AB447467 and AB496719, respectively.

Two supplementary figures are available with the online version of this paper.
sediment (Sakai et al., 2009), fen sediment (Cadillo-Quiroz et al., 2008) and lake sediment (Sakai et al., 2009; Ye et al., 2009), indicating the widespread distribution of Methanolinea-like methanogens.

We previously reported the isolation of a novel methanogen, designated strain TNR$^T$, from rice field soil in Taiwan (Sakai et al., 2009). 16S rRNA gene sequence analysis revealed that the strain has 94.8% 16S rRNA gene sequence similarity with Methanolinea tarda NOBI-1$^T$, suggesting that strain TNR$^T$ might be a member of the genus Methanolinea. In this report, we describe detailed morphological and physiological characteristics and genetic features of strain TNR$^T$ and propose the strain as a representative of a novel species of the genus Methanolinea. In addition, we propose a new family within the order Methanomicrobiales. In recent years, the names of three novel genera within the order Methanomicrobiales have been validly published: the genera Methanolinea (Imachi et al., 2008), Methanosphaerula (Cadillo-Quiroz et al., 2009) and Methanoregula (Bräuer et al., 2011; Yashiro et al., 2011). All those species belong to the family-level clade called the E1/E2 group or Fen Cluster that has long been recognized as an uncultured archaeal group (Bräuer et al., 2006b; Cadillo-Quiroz et al., 2006; Galand et al., 2002; Hales et al., 1996). The 16S rRNA gene based phylogenetic analysis indicates that this clade is distinct from the other families among the order Methanomicrobiales. Therefore, we also propose the family Methanoregulaceae fam. nov., with Methanoregula (Bräuer et al., 2011) as the type genus of this new family.

Strain TNR$^T$ was isolated from an anaerobic, propionate-degrading enrichment culture that was originally obtained from rice field soil of Tainan, Taiwan (Sakai et al., 2009). To obtain the strain in pure culture, we used serial dilution into both liquid and solid media supplemented with H$_2$/CO$_2$ (80:20, v/v; approx. 150 kPa in the head space) or formate (40 mM), using the propionate enrichment as the inoculum. Acetate (1 mM), yeast extract (0.01%, w/v; Difco), vancomycin and ampicillin (50 µg ml$^{-1}$ each) were also added to the cultures. As a result, we obtained a pure culture of strain TNR$^T$ in liquid medium supplemented with H$_2$/CO$_2$. The purity of strain TNR$^T$ was confirmed as previously described (Sakai et al., 2007), with the exception that the 16S rRNA-targeted oligonucleotide probe TNR625 (5‘-TATCCCCGAGCGCCCAT-3‘; positions 125–142 in Escherichia coli) for strain TNR$^T$ was used for fluorescence in situ hybridization (FISH) analysis. The oligonucleotide probe was designed by using the ARB program (Ludwig et al., 2004) and was labelled with Cy3. The stringency of hybridization of the probe was adjusted by adding formamide to the hybridization buffer (35%, v/v). The specificity of the oligonucleotide probe TNR625 was estimated using a pure culture of Halogeometricum borinquense ATCC 700274$^T$ (=JCM 10706$^T$), which contained two mismatches in the probe target site. Non-specific hybridization of probe TNR625 to H. borinquense cells was observed under the hybridization conditions mentioned above.

The basal medium was prepared as previously described (Imachi et al., 2009). The medium consisted of the following components (per litre distilled water): 0.54 g NH$_4$Cl, 0.14 g KH$_2$PO$_4$, 0.2 g MgCl$_2$, 0.6H$_2$O, 0.15 g CaCl$_2$, 2H$_2$O, 2.5 g NaHCO$_3$, 0.3 g Na$_3$S·9H$_2$O, 0.3 g cysteine–HCl, 1 ml trace element solution, 1 ml vitamin solution and 1 ml resazurin solution (1 mg ml$^{-1}$). The trace element solution contained (per litre distilled water): 1.27 g FeCl$_2$, 0.13 g CoCl$_2$, 0.2 g MnCl$_2$, 4H$_2$O, 0.14 g ZnCl$_2$, 0.006 g H$_2$BO$_3$, 0.01 g NiCl$_2$, 0.01 g AlCl$_3$, 0.02 g Na$_2$MoO$_4$, 2H$_2$O, 0.002 g Na$_2$SeO$_3$, 0.003 g Na$_2$WO$_4$, H$_2$O and 0.001 g CuCl$_2$. The vitamin solution was composed of the following vitamins (per litre distilled water): 4.9 mg biotin, 2.7 mg p-aminobenzoic acid, 9.5 mg D-pantothenate (calcium salt), 4.1 mg pyridoxine, HCl, 2.4 mg nicotinic acid, 6.7 mg thiamine, HCl, 4.1 mg lipoic acid, 8.8 mg folic acid, 27.1 mg vitamin B$_12$ and 7.5 mg riboflavin. The standard medium normally contained both 1 mM acetate and 0.01% (w/v) yeast extract. However, those compounds were not added into the medium when growth requirement tests were performed. The cultivations were performed anaerobically at 37 °C under an atmosphere of H$_2$/CO$_2$ (80:20, v/v) or N$_2$/CO$_2$ (80:20, v/v) without shaking. Growth and substrate utilization were determined by monitoring the concentration of methane with a 3200G GC (GL Science) using a thermal conductivity detector. Tests for growth temperature, pH and

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**Fig. 1.** Photomicrographs of strain TNR$^T$ grown on H$_2$/CO$_2$ (approx. 150 kPa in the headspace) medium supplemented with acetate (1 mM) and yeast extract (0.01%, w/v). (a) Phase-contrast and (b) fluorescence micrographs indicating the presence of high levels of coenzyme F$_{420}$ in identical fields. Bars, 10 µm.
salinity range were carried out at 10–60 °C, pH 5.0–8.0 and 0–30 g NaCl l⁻¹. The pH was adjusted at room temperature by adding filter-sterilized HCl or NaOH solution. The medium was routinely monitored with a portable pH meter (HORIBA Twin pH B-212) to determine whether the initial pH conditions had changed during incubation; the pH was readjusted with HCl or NaOH when the initial pH changed significantly. Salinity tests were performed using the same medium described above (which already contains 33 mM Na⁺ and 14 mM Cl⁻). Antibiotic susceptibility was evaluated by using cultures supplemented with antibiotics at final concentrations of 100 μg ml⁻¹. All measurements were performed in triplicate and all incubations were terminated after 3 months.

Cell morphology and motility were examined by phase-contrast microscopy (Olympus BX51F) with a colour CCD camera (Olympus DP71). Susceptibility to lysis was examined by adding SDS to final concentrations of 0.01–2.0 % (w/v) and cell lysis was determined by microscopic observation of cell integrity. The G+C content of the genomic DNA was determined by HPLC as described by Nakagawa et al. (2003). The procedures used for DNA extraction, PCR amplification, cloning and sequencing were as reported elsewhere (Imachi et al., 2006; Sakai et al., 2008). Sequence similarity values were calculated by using the Calculate Matrix function of the ARB program. A phylogenetic tree based on 16S rRNA gene sequences was constructed by using the neighbour-joining method in the ARB program package. To estimate the confidence of the tree topologies, bootstrap resampling analysis (Felsenstein, 1985) with 1000 replicates was performed for the neighbour-joining, maximum-parsimony and maximum-likelihood methods by using MEGA5 software (Tamura et al., 2011).

Cells of strain TNRᵀ were non-motile, rod-shaped, 2.0–6.5 μm long and 0.3 μm wide (Fig. 1). Particularly in late-exponential phase cultures, cultures formed multicellular filaments with lengths of about 100 μm and cocoid cells (about 1 μm diameter). FISH analysis using the strain-specific probe TNR625 identified both cell morphologies as belonging to strain TNRᵀ (Fig. S1, available in IJSEM Online). The cells autofluoresced under epifluorescence microscopy when excited with light near 420 nm in wavelength (Fig. 1). This indicated the presence of high levels of coenzyme F₄₂₀, which is diagnostic for methanogens. The cells resisted disruption in less than 0.1 % (w/v) SDS.

H₂/CO₂ and formate (40 mM) supported growth and methane production by TNRᵀ. The following substrates did not support growth and/or methane production: acetate (20 mM),
Table 1. Comparison of morphological and physiological characteristics of strain TNRT<sup>T</sup>, species within the new family Methanoregulaceae and type species of genera within the order Methanomicrobiales

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Methanoregulaceae</th>
<th>Methanomicrobiaceae</th>
<th>Methanospirillaceae</th>
<th>Methanocorpusculaceae</th>
<th>Unassigned</th>
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<td>Strains: 1, strain TNRT&lt;sup&gt;T&lt;/sup&gt; (data from this study); 2, <em>Methanolinea tarda</em> NOBI-1&lt;sup&gt;T&lt;/sup&gt; (data from Imachi et al., 2008); 3, <em>Methanoregula boonei</em> 6A8&lt;sup&gt;T&lt;/sup&gt; (Bräuer et al., 2006a, 2011); 4, <em>Methanoregula formicica</em> SMSP&lt;sup&gt;3&lt;/sup&gt; (Yashiro et al., 2011); 5, <em>Methanospirausta palustris</em> E1-9c&lt;sup&gt;T&lt;/sup&gt; (Cadillo-Quiroz et al., 2009); 6, <em>Methanomicrobium mobile</em> BP&lt;sup&gt;T&lt;/sup&gt; (Paynter &amp; Hungate, 1968); 7, <em>Methanoculleus bourgensis</em> MS2T (Ollivier et al., 1986); 8, <em>Methanofollis tationis</em> Chile 9T (Zabel et al., 1984); 9, <em>Methanogenium cariaci</em> JR1&lt;sup&gt;T&lt;/sup&gt; (Romesser et al., 1979); 10, <em>Methanolacinia paynteri</em> G-2000T (Rivard et al., 1983); 11, <em>Methanoplanus limicola</em> M3T (Wildgruber et al., 1982); 12, <em>Methanospirillum hungatei</em> JF-1&lt;sup&gt;T&lt;/sup&gt; (Ferry et al., 1974); 13, <em>Methanocorpusculum parvum</em> XIIT (Zellner et al., 1987); 14, <em>Methanocalculus halotolerans</em> SEBR 4845T (Ollivier et al., 1998).</td>
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<tr>
<td></td>
<td>Cell morphology</td>
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<tr>
<td>Cell morphology</td>
<td>Rods*</td>
<td>Rods†</td>
<td>Rods‡</td>
<td>Coccoid</td>
<td>Coccoid</td>
</tr>
<tr>
<td>Cell width/diameter (µm)</td>
<td>0.3</td>
<td>0.7–1.0</td>
<td>0.2–0.3</td>
<td>0.5</td>
<td>0.5-0.8</td>
</tr>
<tr>
<td>Cell length (µm)</td>
<td>2.0–6.5</td>
<td>2.0–8.0</td>
<td>0.8–3.0</td>
<td>1.0–2.6</td>
<td>1.5–2.0</td>
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<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>(weakly)</td>
<td>±</td>
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<tr>
<td>DNA G+C content (mol%)§</td>
<td>56.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>54.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>58.9&lt;sup&gt;d&lt;/sup&gt;</td>
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<tr>
<td>Growth temperature (°C)</td>
<td>Range: 20–40</td>
<td>35–55</td>
<td>10–40</td>
<td>14–35</td>
<td>30–45</td>
</tr>
<tr>
<td>Optimum</td>
<td>57</td>
<td>50</td>
<td>35–37</td>
<td>30–33</td>
<td>40</td>
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<tr>
<td>Growth pH</td>
<td>Range: 6.5–7.4</td>
<td>6.7–8.0</td>
<td>4.5–5.5</td>
<td>7.0–7.6</td>
<td>4.8–6.4</td>
</tr>
<tr>
<td>Optimum</td>
<td>7</td>
<td>7</td>
<td>5.1</td>
<td>7.4</td>
<td>5.7</td>
</tr>
<tr>
<td>Substrate utilization</td>
<td>H&lt;sub&gt;2&lt;/sub&gt;/CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>Formate</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>Secondary</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>Yeast extract</td>
<td>–</td>
<td>–</td>
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<td></td>
<td>Acetate</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td></td>
<td>Coenzyme M</td>
<td>–</td>
<td>–</td>
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*Coccoid and multicellular filamentous cells were observed especially in late-exponential phase culture.
†Cells often formed multicellular filaments longer than 8 µm in the syntrophic propionate-degrading enrichment culture.
‡Coccoid cells were observed in mid- to late-exponential phase culture.
§Determined by: a, HPLC; b, obtained from genome information; c, thermal denaturation; d, buoyant density.
||Coenzyme M was not required for growth, but supplementation with coenzyme M greatly improved the cell density (data from this study).
1-propanol (5 mM), 2-propanol (5 mM), ethanol (5 mM), 1-butanol (5 mM), 2-butanol (5 mM), cyclopentanol (5 mM), methanol (20 mM), dimethylamine (10 mM), trimethylamine (10 mM), and propionate (20 mM). Acetate (1 mM) was required as a carbon source for growth. Yeast extract (0.01 %, w/v) and coenzyme M (0.5 mM) were not required but enhanced growth.

Strain TNR\textsuperscript{T} grew at 20–40 °C (optimal growth at 37 °C), at pH 6.5–7.4 (optimum around pH 7.0) and in the presence of 0–25 g NaCl l\textsuperscript{-1} (optimal growth in 0 g NaCl l\textsuperscript{-1}; growth was inhibited completely in 30 g NaCl l\textsuperscript{-1}). Under optimal conditions (pH 7.0, 37 °C), the doubling time on H\textsubscript{2}/CO\textsubscript{2} medium was approximately 1.2 days, as calculated from the methane production rate. The strain tolerated ampicillin, vancomycin, kanamycin and streptomycin, but not rifampicin, tetracycline or chloramphenicol.

The DNA G+C content of strain TNR\textsuperscript{T} was 56.4 mol%. 16S rRNA gene sequence based phylogenetic analysis showed that strain TNR\textsuperscript{T} is affiliated with the order Methanomicrobiales (Fig. 2). The most closely related strain was Methanolinea tarda NOBI-1\textsuperscript{T} (Imachi et al., 2008), with 16S rRNA and mcrA gene sequence similarities of 94.8 and 86.4 %, respectively.

Strain TNR\textsuperscript{T} and Methanolinea tarda NOBI-1\textsuperscript{T} have similar features. However, the 16S rRNA gene sequence similarity (94.8 %) is in the range of species-level differences (Keswani & Whitman, 2001; Stackebrandt & Goebel, 1994). Thus, strain TNR\textsuperscript{T} and Methanolinea tarda NOBI-1\textsuperscript{T} should be considered as members of the same genus. In addition to the 16S rRNA gene sequence similarities, they have common phenotypic properties. They are hydrogenotrophic methanogens, which can utilize H\textsubscript{2}/CO\textsubscript{2} and formate as substrates. Cell morphology is also similar; both organisms are rod-shaped cells, forming multicellular filaments. However, coccoid type cells were not observed for Methanolinea tarda NOBI-1\textsuperscript{T}. Other differential characteristics between TNR\textsuperscript{T} and Methanolinea tarda NOBI-1\textsuperscript{T} were also observed (Table 1). The temperature ranges for growth differed markedly: strain TNR\textsuperscript{T} was mesophilic whereas Methanolinea tarda NOBI-1\textsuperscript{T} was thermophilic. There are also small differences in the pH range and growth requirements. The pH range for strain TNR\textsuperscript{T} was pH 6.5–7.4 and that for Methanolinea tarda NOBI-1\textsuperscript{T} was pH 6.7–8.0. The supplementation of coenzyme M significantly improved growth of Methanolinea tarda NOBI-1\textsuperscript{T}, but a similar effect was not observed for strain TNR\textsuperscript{T}. On the basis of these physiological and phylogenetic properties, it is proposed that strain TNR\textsuperscript{T} represents a novel species of the genus Methanolinea, Methanolinea mesophila sp. nov.

The members of the genus Methanolinea phylogenetically belong to the family level clade E1/E2 within the order Methanomicrobiales (Fig. 2; alignments of 16S rRNA genes are shown in Fig. S2). This clade also includes two other genera, Methanoregula and Methanosphaerula. The bootstrap values of the 16S rRNA gene-based phylogenetic tree solidly supported the group E1/E2 (Fig. 2). In addition, 16S rRNA gene sequence similarities among strains of species belonging to group E1/E2 (i.e. strain TNR\textsuperscript{T}, Methanolinea tarda NOBI-1\textsuperscript{T}, Methanoregula boonei 6A8\textsuperscript{T}, Methanoregula formicica SMSP\textsuperscript{T} and Methanosphaerula palustris E1-9c\textsuperscript{T}) are in the range 92.8–96.3 %, which are comparable to those among the species of the family Methanomicrobiaceae (89.3–95.1 %), another family within the order Methanomicrobiales (Fig. 2). We therefore propose the status of family for the E1/E2 group and propose the name Methanoregulaceae fam. nov., with Methanoregula as the type genus (type species Methanoregula boonei) of the new family, because Methanoregula boonei 6A8\textsuperscript{T} was the first isolate of the E1/E2 lineage (Bräuer et al., 2006a).

**Description of Methanoregulaceae fam. nov.**

Methanoregulaceae (Me.tha.no.re.gu.lae fam. nov.)

Cells are rod-shaped or coccoid. Use H\textsubscript{2}/CO\textsubscript{2} and sometimes formate for growth and methane production. Acetate is required for growth. Some strains also require yeast extract and coenzyme M for growth. The family belongs to the order Methanomicrobiales. The type genus is Methanoregula.

**Description of Methanolinea mesophila sp. nov.**

*Methanolinea mesophila* (me.so.phi.la. Gr. adj. mesos medium; Gr. adj. philos loving; N.L. fem. adj. mesophila medium-temperature-loving, mesophilic).

Cells are non-motile, rod-shaped, 0.3 μm wide and 2.0–6.5 μm long. Multicellular filamentous (up to around 100 μm) and coccoid (about 1 μm in diameter) cells are also observed, especially in the late-exponential phase cultures. H\textsubscript{2}/CO\textsubscript{2} and formate can be used for growth and methane production. Acetate is required for growth. Yeast extract and coenzyme M enhance growth. Growth occurs at 20–40 °C (optimum at 37 °C), at pH 6.5–7.4 (optimum pH 7.0) and in the presence of NaCl concentrations below 25 g l\textsuperscript{-1}. Cultures are resistant to ampicillin, kanamycin, streptomycin and vancomycin at a concentration of 100 μg ml\textsuperscript{-1}.

The type strain, TNR\textsuperscript{T} (=NBRC 105659\textsuperscript{T}=DSM 23604\textsuperscript{T}), was isolated from rice field soil in Tainan, Taiwan. The DNA G+C content of the type strain is 56.4 mol%.

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