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Abstract

A novel mesophilic, hydrogenotrophic methanogen, designated strain TNRT, 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 mm long by 0.3 mm wide. Filamentous (up to about 100 mm) and coccoid (about 1 mm in diameter) cells were also observed in cultures in the late exponential phase of growth. Strain TNRT 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 TNRT 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 TNRT 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 TNRT 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 TNRT (5NBRC 105659T5DSM 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 \( \text{H}_2/\text{CO}_2 \)-using methanogenic archaeon (Imachi et al., 2008). The genus currently consists of only one species, Methanolinea tarda, the type strain of which, NOBI-1\(^1\), 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; Díaz et al., 2006; Imachi et al., 2008; Lykidis et al., 2011; Narihiro et al., 2009; Sakai et al., 2009; Yashiro et al., 2011), marine
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 Methanolinella-like methanogens.

We previously reported the isolation of a novel methanogen, designated strain TNRT, 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 Methanolinella tarda NOBI-1, suggesting that strain TNRT might be a member of the genus Methanolinella. In this report, we describe detailed morphological and physiological characteristics and genetic features of strain TNRT and propose the strain as a representative of a novel species of the genus Methanolinella. 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 Methanolinella (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 TNRT 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 H₂/CO₂ (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 TNRT in liquid medium supplemented with H₂/CO₂. The purity of strain TNRT was confirmed as previously described (Sakai et al., 2007), with the exception that the 16S rRNA-targeted oligonucleotide probe TNR625 (5'-TATCCCGGACGCCCAT-3'; positions 125–142 in Escherichia coli) for strain TNRT 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 700274T (= JCM 10706T), 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 NH₄Cl, 0.14 g KH₂PO₄, 0.2 g MgCl₂, 6H₂O, 0.15 g CaCl₂, 2H₂O, 2.5 g NaHCO₃, 0.3 g Na₂S. 9H₂O, 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 FeCl₂, 0.13 g CoCl₂, 0.2 g MnCl₂, 4H₂O, 0.14 g ZnCl₂, 0.006 g H₂BO₃, 0.01 g NiCl₂, 0.01 g AlCl₃, 0.02 g Na₂MoO₄. 2H₂O, 0.002 g Na₂SeO₃, 0.003 g Na₂WO₄, H₂O and 0.001 g CuCl₂. 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 lipic acid, 8.8 mg folic acid, 27.1 mg vitamin B₁₂ 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 H₂/CO₂ (80:20, v/v) or N₂/CO₂ (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 TNRT grown on H₂/CO₂ (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 F₄₂₀ in identical fields. Bars, 10 μm.
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 \(\mu\)g l\(^{-1}\).

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 TNR\(^T\) were non-motile, rod-shaped, 2.0–6.5 \(\mu\)m long and 0.3 \(\mu\)m wide (Fig. 1). Particularly in late-exponential phase cultures, cultures formed multicellular filaments with lengths of about 100 \(\mu\)m and coccoid cells (about 1 \(\mu\)m diameter). FISH analysis using the strain-specific probe TNR625 identified both cell morphologies as belonging to strain TNR\(^T\) (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.

H\(_2/\)CO\(_2\) and formate (40 mM) supported growth and methane production by TNR\(^T\). The following substrates did not support growth and/or methane production: acetate (20 mM),...
Table 1. Comparison of morphological and physiological characteristics of strain TNR\(^T\), species within the new family Methanoregulaceae and type species of genera within the order Methanomicrobiales

Strains: 1, strain TNR\(^T\) (data from this study); 2, Methanolinea tarda NOBI-1\(^T\) (data from Imachi et al., 2008); 3, Methanoregula boonei 6A8\(^T\) (Bräuer et al., 2006a, 2011); 4, Methanoregula formicica SMSP\(^T\) (Yashiro et al., 2011); 5, Methanospirillum hungatii E1-9c\(^T\) (Cadillo-Quiroz et al., 2009); 6, Methanocorpusculum parvum XIIT (Zellner et al., 1987); 7, Methanocalculus halotolerans SEBR 4845\(^T\) (Ollivier et al., 1998).

2, Negative; +, positive; ±, species-dependent; NR, not reported.

<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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<tbody>
<tr>
<td>Cell morphology</td>
<td>Rods*</td>
<td>Rods†</td>
<td>Rods‡</td>
<td>Cocoid</td>
<td>Curved rod</td>
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<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>
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<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>
</tr>
<tr>
<td>Motility</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DNA G+C content (mol%)§</td>
<td>56.4(^a)</td>
<td>56.3(^a)</td>
<td>54.3(^b)</td>
<td>56.2(^a)</td>
<td>58.9(^b)</td>
</tr>
<tr>
<td>Optimum</td>
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<td>50</td>
<td>35–37</td>
<td>30–33</td>
<td>30</td>
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<tr>
<td>Growth pH</td>
<td>Range</td>
<td>6.5–7.4</td>
<td>6.7–8.0</td>
<td>4.5–5.5</td>
<td>7.0–7.6</td>
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<td>Optimum</td>
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<td>7</td>
<td>5.1</td>
<td>7.4</td>
<td>5.7</td>
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<td>+</td>
<td>+</td>
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<td>Yeast extract</td>
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<td>+</td>
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<td>Coenzyme M</td>
<td>–</td>
<td>–</td>
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<td></td>
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</table>

*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.
‡Coccolid 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), methylamine (10 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$^T$ 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$^{-1}$ (optimal growth in 0 g NaCl l$^{-1}$; growth was inhibited completely in 30 g NaCl l$^{-1}$). Under optimal conditions (pH 7.0, 37 °C), the doubling time on H$_2$/CO$_2$ 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$^T$ was 56.4 mol%. 16S rRNA gene sequence based phylogenetic analysis showed that strain TNR$^T$ is affiliated with the order *Methanomicrobiales* (Fig. 2). The most closely related strain was *Methanolinea tarda* NOBI-1$^T$ (Imachi et al., 2008), with 16S rRNA and mcrA gene sequence similarities of 94.8 and 86.4 %, respectively.

Strain TNR$^T$ and *Methanolinea tarda* NOBI-1$^T$ 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$^T$ and *Methanolinea tarda* NOBI-1$^T$ 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$_2$/CO$_2$ 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$^T$. Other differential characteristics between TNR$^T$ and *Methanolinea tarda* NOBI-1$^T$ were also observed (Table 1). The temperature ranges for growth differed markedly: strain TNR$^T$ was mesophilic whereas *Methanolinea tarda* NOBI-1$^T$ was thermophilic. There are also small differences in the pH range and growth requirements. The pH range for strain TNR$^T$ was pH 6.5–7.4 and that for *Methanolinea tarda* NOBI-1$^T$ was pH 6.7–8.0. The supplementation of coenzyme M significantly improved growth of *Methanolinea tarda* NOBI-1$^T$, but a similar effect was not observed for strain TNR$^T$. On the basis of these physiological and phylogenetic properties, it is proposed that strain TNR$^T$ 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$^T$, *Methanolinea tarda* NOBI-1$^T$, *Methanoregula boonei* 6A8$^T$, *Methanoregula formicica* SMSP$^T$ and *Methanosphaerula palustris* E1-9c$^T$) 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$^T$ was the first isolate of the E1/E2 lineage (Bräuer et al., 2006a).

**Description of Methanoregulaceae fam. nov.**

*Methanoregulaceae* (Me.tha.no.re.gu.la.ce.ae N.L. fem. n. *Methanoregula* type genus of the family; suff. -aceae ending to donate a family; N.L. fem. pl. n. *Methanoregulaceae* the *Methanoregula* family).

Cells are rod-shaped or coccoid. Use H$_2$/CO$_2$ 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$_2$/CO$_2$ 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$^{-1}$. Cultures are resistant to ampicillin, kanamycin, streptomycin and vancomycin at a concentration of 100 μg ml$^{-1}$.

The type strain, TNR$^T$ (=NBRC 105659$^T$=DSM 23604$^T$), 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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**References**


