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*

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A novel mesophilic, hydrogenotrophic methanogen, designated strain TNR^T, 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 TNR^T 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 H_2/CO_2 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 TNR^T was related most closely to *Methanolinea tarda* NOBI-1^T; levels of sequence similarities were 94.8 and 86.4 %, respectively. The 16S rRNA gene sequence similarity indicates that strain TNR^T and *M. tarda* NOBI-1^T 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 TNR^T 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 TNR^T (=NBRC 105659^T=DSM 23604^T). 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 H_2/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^T, 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

Abbreviation: FISH, fluorescence *in situ* hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *mcrA* gene sequences of strain TNR^T 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′-TATCCCCCGGACGCCCAT-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_4Cl, 0.14 g KH_2PO_4, 0.2 g $MgCl_2 . 6H_2O$, 0.15 g $CaCl_2 . 2H_2O$, 2.5 g $NaHCO_3$, 0.3 g $Na_2S . 9H_2O$, 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 $MnCl_2 . 4H_2O$, 0.14 g $ZnCl_2$, 0.006 g H_3BO_3, 0.01 g $NiCl_2$, 0.01 g $AlCl_3$, 0.02 g $Na_2MoO_4 . 2H_2O$, 0.002 g Na_2SeO_3, 0.003 g $Na_2WO_4 . H_2O$ 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

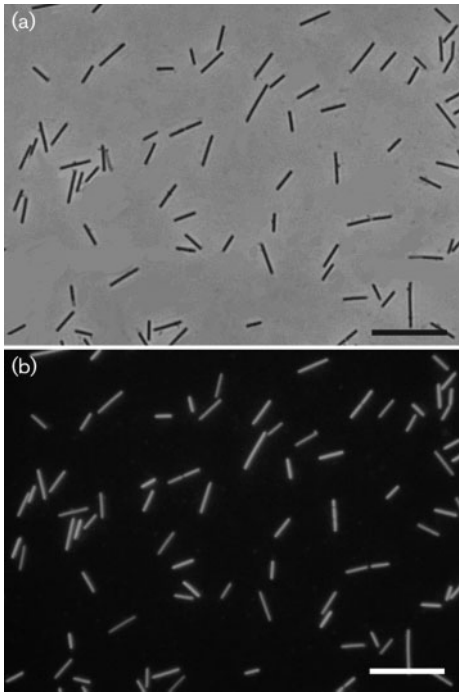

Fig. 1. Photomicrographs of strain TNR^T grown on H_2/CO_2 (approx. 150 kPa in the headspace) medium supplemented with acetate (1 mM) and yeast extract (0.01 %, w/v). (a) Phase-contrast and (b) fluorescence micrographs indicating the presence of high levels of coenzyme F_{420} in identical fields. Bars, 10 μm.

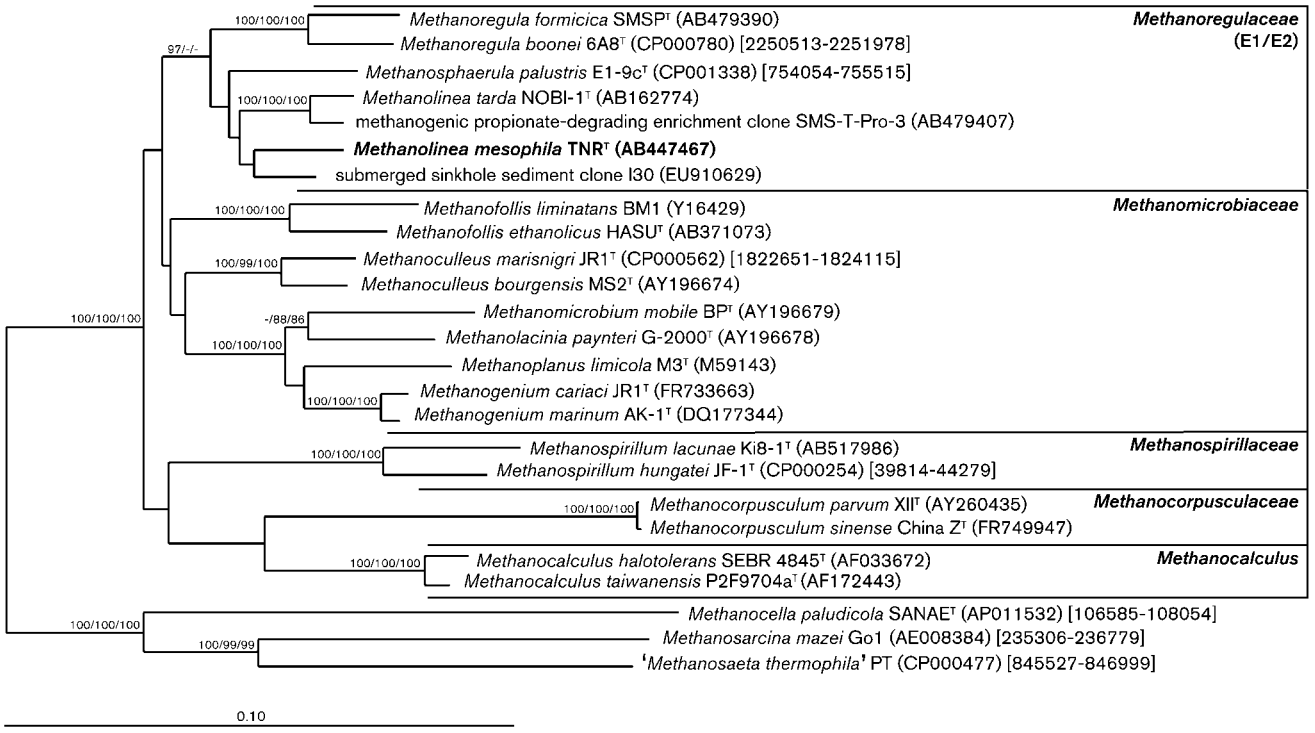

Fig. 2. Phylogenetic tree of the order *Methanomicrobiales* based on comparative analyses of 16S rRNA gene sequences, showing the placement of strain TNR^T. The tree was calculated on a subset of 25 nearly full-length 16S rRNA gene sequences by neighbour-joining analysis in combination with filters, which considered at least 50 % conserved regions of the 16S rRNA sequences to exclude the influence of highly variable positions. For sequences taken from complete genomic sequences, their position on the genome is indicated in brackets. Bootstrap values were obtained from the neighbour-joining/maximum-parsimony/maximum-likelihood methods based on 1000 replicates; –, <50 %. Bar, 0.10 changes per nucleotide sequence position.

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 TNR^T 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 coccoid cells (about 1 μm 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 F_{420}, 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, *Methanosphaerula palustris* E1-9c^T (Cadillo-Quiroz *et al.*, 2009); 6, *Methanomicrobium mobile* BP^T (Paynter & Hungate, 1968); 7, *Methanoculleus bourgensis* MS2^T (Ollivier *et al.*, 1986); 8, *Methanofollis tationis* Chile 9^T (Zabel *et al.*, 1984); 9, *Methanogenium cariaci* JR1^T (Romesser *et al.*, 1979); 10, *Methanolacinia paynteri* G-2000^T (Rivard *et al.*, 1983); 11, *Methanoplanus limicola* M3^T (Wildgruber *et al.*, 1982); 12, *Methanospirillum hungatei* JF-1^T (Ferry *et al.*, 1974); 13, *Methanocorpusculum parvum* XII^T (Zellner *et al.*, 1987); 14, *Methanocalculus halotolerans* SEBR 4845^T (Ollivier *et al.*, 1998). −, Negative; +, positive; ±, species-dependent; NR, not reported.

Characteristic	*Methanoregulaceae*					*Methanomicrobiaceae*						*Methano-spirillaceae*	*Methano-corpusculaceae*	Unassigned
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Cell morphology	Rods*	Rods†	Rods and irregular coccoid	Rods‡	Coccoid	Curved rod	Irregular coccoid	Irregular coccoid	Irregular coccoid	Irregular rod	Plate-shaped	Curved rod	Irregular coccoid	Irregular coccoid
Cell width/diameter (μm)	0.3	0.7–1.0	0.2–0.3	0.5	0.5–0.8	0.6–0.7	1.0–2.0	1.5–3.0	>2.6	0.6	1.6–2.8	0.4–0.5	>1.0	0.8–1.0
Cell length (μm)	2.0–6.5	2.0–8.0	0.8–3.0	1.0–2.6		1.5−2.0				1.5–2.5	1.5	7.4–20		
Motility	−	−	−	−	−	+ (weakly)	−	±	−	−	+ (weakly)	+ (weakly)	+ (weakly)	NR
DNA G+C content (mol%)§	56.4[a]	56.3[a]	54.5[b]	56.2[a]	58.9[c]	48.8[d]	59[d]	54[c]	51.6[d]	44.9[d]	47.5[c]	45[d]	48.5[c]	55[a]
Growth temperature (°C)														
Range	20–40	35–55	10–40	10–40	14–35	30–45	NR	25–45	NR	20–45	17–41	NR	20–45	25–45
Optimum	37	50	35–37	30–33	30	40	37	37–40	20–25	45	40	30–37	37	38
Growth pH														
Range	6.5–7.4	6.7–8.0	4.5–5.5	7.0–7.6	4.8–6.4	5.9–7.7	5.5–8.0	6.3–8.8	NR	6.6–7.3	NR	NR	6.0–8.0	7.0–8.4
Optimum	7	7	5.1	7.4	5.7	6.1–6.9	6.7	7	6.8–7.3	7	6.5–7.5	6.6 – 7.4	6.8–7.5	7.6
Substrate utilization														
H_2/CO_2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Formate	+	+	−	+	+	+	+	+	+	−	+	+	+	+
Secondary alcohols	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Growth requirements														
Yeast extract	−	+	+	+	−	+	−	+	+	−	−	−	+	−
Acetate	+	+	+	+	+	+	+	+	+	+	+	−	+	+
Coenzyme M	−	−‖	+	−	+	−	NR	NR	NR	NR	NR	NR	NR	NR

*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), 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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