Methanolipina tarda gen. nov., sp. nov., a methane-producing archaeon isolated from a methanogenic digester sludge

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A novel methane-producing archaeon, strain NOBI-1T was isolated from an anaerobic, propionate-degradation enrichment culture, which was originally obtained from a mesophilic methanogenic sludge digesting municipal sewage sludge. Cells were non-motile, rod-shaped, 0.7–1.0 μm by 2.0 μm, and formed multicellular filaments longer than 8 μm. Growth was observed between 35 and 55 °C (optimum 50 °C) and pH 6.7 and 8.0 (optimum pH 7.0). The G+C content of the genomic DNA was 56.3 mol%. The strain utilized H2 and formate for growth and methane production. Based on comparative sequence analyses of the 16S rRNA gene and mcrA gene (encoding the alpha subunit of methyl-coenzyme M reductase, a key enzyme in the methane-production pathway), strain NOBI-1T was affiliated with the order Methanomicrobiales, but it was significantly distant from any other known species within the order. The most closely related species based on 16S rRNA and mcrA gene sequence similarity were respectively ‘Candidatus Methanoregula boonei’ (93.7 % 16S rRNA gene sequence similarity) and Methanocorpusculum parvum (74.2 % deduced McrA amino acid sequence similarity to the type strain). These phenotypic and genetic properties justified the creation of a novel species of a new genus for the strain, for which we propose the name Methanolipina tarda gen. nov., sp. nov. The type strain of Methanolipina tarda is strain NOBI-1T (=DSM 16494T =JCM 12467T =NBRC 102358T).

Abbreviation: FISH, fluorescence in situ hybridization.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain NOBI-1T is AB162774 (other 16S rRNA gene clonal sequences have been deposited as accession numbers AB233209, AB233210 and AB233209–AB233302). The GenBank/EMBL/DDBJ accession numbers for the mcrA gene sequences of strains NOBI-1T and SANAE are respectively AB300466 and AB300467.

Details of 16S rRNA gene phylotypes obtained from the sludge, information on relative abundance of various groups of organisms and a graph of methane production by strain NOBI-1T are available as supplementary material with the online version of this paper.
Recently, we reported the isolation of a novel methanogenic archaeon, designated strain NOBI-1T, from a mesophilic, methanogenic sludge digesting municipal sewage sludge (Sakai et al., 2007). To cultivate the strain, we used the 'co-culture' method, which takes advantage of low but predictable H₂ evolution rates from a companion syntrophic bacterium to enrich and isolate the methanogen. The point was that the co-culture method mimics the in situ substrate (H₂) flow in natural environments by adding a propionate-oxidizing H₂-producing syntroph and propionate as the H₂ source for methanogens. In the previous study, we described the isolation of the strain in pure culture, but we did not characterize the strain fully. In this report, we describe the detailed isolation procedure, morphological and physiological characteristics and genetic features of strain NOBI-1T and propose this strain as a member of a novel species of a new genus.

A methanogenic sludge sample was obtained from a municipal sewage plant at Nagoaka, Niigata, Japan. After sampling, the sludge was washed immediately with phosphate buffer (10 mM, pH 7.2) and homogenized briefly to inoculate into a primary enrichment culture medium. The medium for cultivation of strain NOBI-1T was prepared as described previously (Sekiguchi et al., 2000). Enrichment cultures were incubated anaerobically at 37 °C. After isolation of the strain, all axenic incubations were performed at 50 °C in 50 ml serum vials containing 20 ml medium (pH at 25 °C, 7.2) under an atmosphere of N₂/CO₂ (80 : 20, v/v) without shaking, unless otherwise mentioned. The serum vials were sealed with butyl rubber stoppers and aluminium crimp seals. To monitor whether the media were kept under anaerobic conditions, resazurin was added to the medium as a redox indicator. In the nutritional tests, substrates added to the vials were neutralized prior to inoculation. Growth and substrate utilization were determined by monitoring turbidity by optical density at 600 nm and the production of methane. All incubations for growth/substrate utilization tests were performed at 50 °C for over 3 months. During the incubation for 3 months, all media had been kept under anaerobic conditions (since the resazurin remained clear in colour). Effects of pH, temperature and NaCl concentration on growth of strain NOBI-1T were determined in medium containing 0.01 % yeast extract and 1 mM acetate in the presence of approx. 150 kPa H₂. For determination of the optimal pH for growth, the pH of the hydrogen-supplemented medium was adjusted at room temperature to 5.5–8.0 by adding HCl or NaOH solution under a 100 % N₂ atmosphere prior to inoculation. To determine the temperature range for growth, cultures were incubated at 25–60 °C (pH 7.2). To evaluate the effect of NaCl concentration on growth, an autoclaved NaCl solution was added to the medium to give final concentrations of 1–30 g l⁻¹. Effects of antibiotics on growth were evaluated with a culture (0.01 % yeast extract, 1 mM acetate, 150 kPa H₂) supplemented with each antibiotic at the final concentration of 100 µg ml⁻¹. All measurements were performed in triplicate.

Cell morphology was examined under a fluorescence microscope (Olympus BX50F). The Gram-staining reaction was performed by Hucker’s method (Doetsch, 1981). The susceptibility to lysis was checked by adding SDS with final concentrations of 0.01–1.0 %, and cell lysis was determined by microscopic observation of cell integrity. Transmission electron microscopy was performed with a Hitachi H7000 transmission electron microscope as described previously (Hattori et al., 2000). Short-chain fatty acids, alcohols, methane, H₂ and carbon dioxide were measured as described previously (Imachi et al., 2000, 2002, 2006). The G + C content of the genomic DNA was determined as described by Kamagata & Mikami (1991).

All procedures for DNA extraction, 16S rRNA gene-based cloning and sequencing were performed previously (Imachi et al., 2006). In the PCR amplification, we used the primer pair Ar109f (Großkopf et al., 1998) and 1490R (Weisburg et al., 1991) or EUB338* (Amann et al., 1990; Daims et al., 1999; Hatamoto et al., 2007) and 1490R for the construction of 16S rRNA gene-based archaeal and bacterial clone libraries, respectively. We also used primers Arch21F (DeLong, 1992) and 1490R to obtain the nearly full-length 16S rRNA gene of the isolate. For PCR amplification of the mcrA gene, we used primers ME1/ME2 (Hales et al., 1996). Comparative 16S rRNA gene sequence analysis was performed as described elsewhere (Imachi et al., 2006). 16S rRNA gene sequence similarity values were calculated using the Calculate Matrix function of the ARB program with Jukes and Cantor correction (Jukes & Cantor, 1969; Ludwig et al., 2004). A deduced McrA amino acid sequence-based phylogenetic tree was constructed by the neighbour-joining method implemented in the ARB program with 164 amino acid positions and percentage of acceptance mutations (PAM) distance correction. For both phylogenetic trees, bootstrap resampling analysis for 1000 replicates was performed with the neighbour-joining, maximum-parsimony and maximum-likelihood methods to estimate the confidence of tree topologies as described previously (Sekiguchi et al., 2006). Quantitative PCR analysis was performed as described previously (Imachi et al., 2006). For the quantitative analysis, the following 16S rRNA gene-targeted PCR primer sets were used: 519f and 907r (Lane, 1991; Stubbner, 2002) for the domain Bacteria, Ar109f and Ar912r (Großkopf et al., 1998) for the domain Archaea and NOBI109f (5′-ACTGCTCAAGTAAACAGT-3′) and NOBI633 (5′-GATTGCCAGTTTCTCCTG-3′) for strain NOBI-1T and its relatives. Primer NOBI109f was a slightly modified version of the primer reported by Großkopf et al. (1998) to match perfectly with the 16S rRNA gene sequence of strain NOBI-1T. Primer NOBI633 was designed using the ARB program (Ludwig et al., 2004). For construction of standard templates, we used dilution series of PCR-amplified 16S rRNA genes of Escherichia coli strain JM109 (TaKaRa Bio Inc.), Methanobacterium bryantii DSM 863T and strain NOBI-1T, which were obtained with bacterial universal primer pair 8f/1490R.
(Weisburg et al., 1991) or the archaeal universal primer pair Arch21F/1490R. The optimal PCR conditions were determined by changing the Mg^{2+} concentration in the PCR buffer (4 mM for 519f/907r, 3 mM for Ar109f/Ar912r and 3 mM for NOBI109f/NOBI633) and the PCR annealing temperature for each primer set (50 °C for 519f/907r, 52 °C for Ar109f/Ar912r and 64 °C for NOBI109f/NOBI633). The quantitative PCR conditions were as follows: initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 0 s, 10 s annealing at the optimal temperatures mentioned above and extension at 72 °C for 30, 35 and 21 s for primer pairs 519f/907r, Ar109f/Ar912r and NOBI109f/ NOBI633, respectively. Fluorescence in situ hybridization (FISH) was done according to the method described by Sekiguchi et al. (1998). To detect strain NOBI-1^T cells by FISH, we used probe NOBI633 that was also used as a primer for the quantitative real-time PCR analysis. To evaluate the specificity of probe NOBI633, cells of Methanobacterium bryantii DSM 863^T, Methanospirillum hungatei DSM 864^T and Methanoculleus palmolei DSM 4273^T were used as reference organisms. Hybridization stringency of the probe was adjusted by changing the formamide concentration in the hybridization buffer; the stringent condition for the probe NOBI633 was determined to be 10 % (v/v) formamide in the hybridization buffer. The oligonucleotide probe was labelled with Cy-3.

Our preliminary analyses of 16S rRNA gene-based cloning and quantitative PCR revealed that the methanogenic sludge used as the inoculum contained an uncultured archaeal group within the order Methanomicrobiales, possibly methanogens, as approximately 10 % of the total archaeal population (Fig. 1, phylotype ND-3; Supplementary Tables S1 and S2, available in IJSEM Online). To cultivate the uncultured organisms, the sludge was incubated anaerobically with 20 mM propionate. Cell growth and methane production along with propionate degradation in the culture occurred after 2 months of incubation. During the cultivation, the H₂ partial pressure in the culture was determined to be in the range of 16–28 Pa. The culture was successively transferred into fresh medium every 60–80 days [10 % inoculum (v/v)]. Microscope observation showed that the propionate enrichment culture after five repeated transfers contained at least three morphologically distinct organisms: (i) blunted-rod-shaped, F₄₂₀-autofluorescent methanogen-like cells, (ii) oval rods and (iii) coccoid-shaped microbes as a minor population (Fig. 2a). These findings suggested that propionate degradation was carried out by syntrophic association between propionate-degrading microbes and hydrogenotrophic methanogens. To identify the microorganisms present in the enrichment culture, 16S rRNA gene-based clone analysis was performed. The analysis indicated that all ten of the archaeal rRNA gene clones were identical (Fig. 1; phylotype ND-Pro-Arc-1) and had the same sequence as a phylotype recovered from the original methanogenic sludge. The phylotype was affiliated with the uncultured archaeal group within the order Methanomicrobiales. On the other hand, the most frequently retrieved bacterial phylotype (8/10 clones) was closely related to Syntrophobacter fumaroxidans (GenBank accession no. AB233309; 98 % sequence similarity), a mesophilic, syntrophic propionate-oxidizing deltaproteobacterial strain.

![Fig. 1. Phylogenetic tree of the order Methanomicrobiales based on comparative analyses of 16S rRNA gene sequences, showing the placement of the isolated methanogenic archaeon strain NOBI-1^T (Methanolinea tarda). The tree was calculated based on a distance matrix analysis of 16S rRNA gene sequences (neighbour-joining tree). Three 16S rRNA gene sequences of organisms belonging to the class Thermoplasmata [Picrophilus oshimae KAW2/2^T (GenBank accession no. XB4901), clone WCHD3-02 (AF050616) and clone pMC2A24 (AB019736)] were used to root the tree (not shown). Branching points that supported probabilities above 90 % by all the analyses [based on 1000 replicates, estimated using neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) methods] are indicated by solid circles, and nodes with open circles indicate >70 % bootstrap probability support by the three analyses. Accession numbers are shown in parentheses. Bar, 0.1 nucleotide changes per sequence position.](image-url)
species (Harmsen et al., 1998). The remaining bacterial phylotype (2/10 clones) was affiliated with the phylum Spirochaetes, and its most closely related cultured species was Spirochaeta stenostrepta (GenBank accession no. AB233310; 87% sequence similarity). FISH analysis using the specific probe NOBI633 for the archaeal phylotype also confirmed that NOBI633-probe-reactive, rod-shaped cells were actually present as a significant population in the culture (data not shown). To isolate methanogenic archaeal cells axenically in the propionate enrichment culture, we used substrates generally utilized by methanogens, i.e. H₂ (approx. 150 kPa) and formate (40 mM). Colony isolation was at first attempted by the roll-tube method, but the attempt was unsuccessful. Purification of the methanogen was, therefore, attempted by repetitive serial dilutions in liquid medium. The highest dilution with growth (usually dilution 10⁻³ to 10⁻⁴) was always used for subculturing. After seven successive transfers, we obtained a pure culture of the methanogenic archaeon, strain NOBI-1ᵀ, in liquid medium supplemented with H₂ (Fig. 2b). The purity of strain NOBI-1ᵀ was demonstrated by the failure to grow in the following media at 37 and 55 ℃: (i) thioglycollate medium (Difco) containing approx. 150 kPa H₂/CO₂ (in the head space) and 10 mM sulphate; (ii) thioglycollate medium containing 20 mM lactate and 10 mM sulphate; (iii) thioglycollate medium containing 10 mM sucrose, 10 mM glucose, 10 mM cellobiose and 10 mM xylose and (iv) AC medium (Difco). Moreover, we also tested the purity by 16S rRNA gene-based cloning analysis using the archaeal universal primer pair Ar109f/1490R. Thirty clones was selected randomly and sequenced. All 30 clonal sequences were identical and had the same sequence as strain NOBI-1ᵀ. Also, we performed FISH with 16S rRNA-targeted oligonucleotide probe NOBI633 specific for strain NOBI-1ᵀ, and we confirmed that all cells hybridized with the probe (data not shown). In addition, we also evaluated purity by the failure to recover bacterial 16S rRNA gene amplifications by PCR with a universal bacterial primer pair EUB338f/1490R. The results of these molecular surveys also indicated that the NOBI-1ᵀ culture was axenic.

Cells of strain NOBI-1ᵀ were straight rods with blunt-ends, non-motile, 0.7–1.0 μm wide and 2.0 μm long (Fig. 2b). The cells often formed multicellular filaments longer than 8 μm in the syntrophic propionate-degrading enrichment culture (Fig. 2a). Electron micrographs showed that the strain had a sheath-like structure (Fig. 2c). The cells were Gram-reaction negative. The cells were susceptible to lysis with 0.1% SDS (w/v), suggesting the presence of a proteinaceous cell wall (Boone & Whitman, 1988).

The strain was strictly anaerobic, since its growth was inhibited completely in the presence of trace quantities of oxygen [0.1 and 0.2% O₂ (v/v)]. Yeast extract and acetate were required for growth. H₂ and formate (40 mM) supported growth and methane production. In the H₂ culture, we observed that H₂ was converted to methane stoichiometrically, which is explained by the equation 4H₂ + CO₂ → CH₄ + 2H₂O (Supplementary Fig. S1). The following substrates did not support growth and methane production: pyruvate (20 mM), lactate (20 mM), acetate (20 mM), propionate (20 mM), trimethylamine (10 mM),
dimethylamine (10 mM), methylamine (10 mM), cyclopentanol (20 mM), ethanol (5 mM), methanol (20 mM), 1-propanol (5 mM), 2-propanol (5 mM), 1-butanol (5 mM) and 2-butanol (5 mM).

The optimum growth temperature of strain NOBI-1T was 50 °C, although the first enrichment culture was incubated at 37 °C. No growth occurred below 30 °C or above 60 °C over 3 months of incubation. The pH range for growth of the strain was estimated to be pH 6.7–8.0, with an optimum of pH 7.0. Under optimum conditions (pH 7.0, 50 °C), the specific growth rate on H₂ medium was approx. 0.17 day⁻¹, which was calculated based on measurement of optical density at 600 nm. To the best of our knowledge, this specific growth rate is the slowest so far reported among known methanogens. The strain was basically a freshwater organism, but could grow at NaCl concentrations up to 15 g l⁻¹. Strain NOBI-1T tolerated ampicillin, penicillin G, vancomycin, kanamycin and streptomycin. Rifampicin, tetracycline, monensin and chloramphenicol inhibited cell growth completely.

The G+C content of total DNA in strain NOBI-1T was 56.3 mol%. For strain NOBI-1T, 1433 bp of the 16S rRNA gene sequence was determined. Comparative 16S rRNA gene sequence analysis showed that strain NOBI-1T was affiliated with the order Methanomicrobiales (Fig. 1). The closest relative of strain NOBI-1T was ‘Candidatus Methanoregula boonei’ (sequence similarity 93.7 %; Fig. 1), a recently described acidophilic hydrogenotrophic methanogen isolated from an acidic peat bog (Brauer et al., 2006). In addition to the 16S rRNA gene sequence-based analysis, we determined the sequence of part of the mcrA gene (763 bp) and then constructed a molecular phylogenetic tree based on deduced amino acid sequences of mcrA genes (Fig. 3). The McrA amino acid sequence-based tree also indicated that strain NOBI-1T was a member of the order Methanomicrobiales. However, we could not confirm an mcrA gene-based relationship between strain NOBI-1T and ‘Candidatus Methanoregula boonei’ because of the lack of information about the mcrA gene of the latter. The closest relative on the basis of the McrA amino acid sequence was Methanocorpusculum parvum XII (sequence similarity 74.2 %) (Zellner et al., 1987).

Based on its physiological and molecular phylogenetic traits, strain NOBI-1T is certainly considered to be a member of the order Methanomicrobiales. As shown in Table 1, the members of the order have a wide variety of phenotypic characteristics, e.g. cell morphology and substrate usage, with the exception that methane production from H₂ is a common physiological property. Therefore, it is difficult to distinguish the members of the order based solely on their physiological properties and thus molecular phylogeny is a key indicator for the taxonomic identification of its members (Asakawa & Nagaoka, 2003; Boone et al., 1993; Maestrojúan et al., 1990; Rouvière et al., 1992; Spring et al., 2005; Zellner et al., 1989, 1999).

The 16S rRNA gene-based phylogenetic analysis presented in this study indicated that strain NOBI-1T was most closely related to ‘Candidatus Methanoregula boonei’ (Fig. 1). The similarity value between the organisms is 93.7 %, which is in the range of genus-level differences among described genera belonging to the order [e.g. the differences between the genera Methanoplanus and Methanomicibrium (93.9 %) and between the genera Methanofollis and Methanoculleus (93.5 %)]. In addition, our isolate and ‘Candidatus Methanoregula boonei’ have several clearly different physiological features; i.e. cell morphology, temperature and pH range for growth and substrate utilization (Table 1). These results justified the creation of a new genus for strain NOBI-1T.

Based on these phenotypic and phylogenetic findings, we propose strain NOBI-1T as a member of a novel genus and species with the name Methanolinea tarda gen. nov., sp. nov.

**Description of Methanolinea gen. nov.**

*Methanolinea* [Me.tha.no.li.nea’ a. N.Gr. n. methane (from N.Gr. n. meth(y)l and chemical suffix -ane) methane; L. fem. n. linea line; N.L. fem. n. Methanolinea a methane-producing, line-shaped morphotype].
Rod-shaped cells with blunt-ends. Often form multicellular filaments. Methanogenic and strictly anaerobic members of the order **Methanomicrobiales**, phylum **Euryarchaeota**, domain **Archaea**. Can use H₂ or formate for growth and methane production. The type species is **Methanolinea tarda**.

**Description of Methanolinea tarda** sp. nov.

*Methanolinea tarda* (L. fem. adj. *tarda* slow, referring to its slow growth).

Strictly anaerobic. Cells are non-motile and rod-shaped, 0.7–1.0 µm wide and 2.0 µm long. Cells often form multicellular filaments longer than 8 µm in syntrophic propionate-degrading enrichment culture. Gram-reaction negative. H₂ and formate can be used for growth and methane production. Yeast extract and acetate are required for growth. The temperature range for growth is 35–55 °C (optimum 50 °C). The pH range for growth is 6.7–8.0 (optimum pH 7.0). Under optimum conditions, the growth rate is approx. 0.17 day⁻¹. Growth occurs in the presence of 0–1.5 % NaCl but does not occur in the presence of 2.0 % NaCl. The G + C content of the DNA of the type strain is 56.3 mol%.

The type strain, NOBI-1T (=DSM 16494T =JCM 12467T =NBRC 102358T), was isolated from a mesophilic, methanogenic sludge digesting sewage sludge.

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