Methanobacterium kanagiense sp. nov., a hydrogenotrophic methanogen, isolated from rice-field soil

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A pure culture of an obligately anaerobic, hydrogenotrophic, methanogenic archaeon, designated strain 169^T, which grows with hydrogen and carbon dioxide as the sole energy and carbon sources, was isolated from an anaerobic propionate-oxidizing enrichment culture originally obtained as an inoculant from rice-field soil in Japan. Cells of strain 169^T were non-motile, Gram-reaction-variable and rod-shaped or slightly curved rods with rounded ends (1.6–5.0×0.35–0.5 µm). Strain 169^T had fimbriae at both ends of the cell (up to ~10 per cell) but did not possess flagella. Ultrathin sections showed a single-layered, electron-dense cell wall about 6 nm thick, which is typical of Gram-positive bacteria. Growth was observed at 15 °C–45 °C (optimum 40 °C), at pH 6.5–9.6 (optimum pH 7.5–8.5) and in 0–70 g NaCl l⁻¹ (0–1.2 M) (optimum 5 g NaCl l⁻¹; 0.086 M). Strain 169^T utilized only hydrogen and carbon dioxide as energy and carbon sources. The DNA G+C content was 39.3 mol%. The results of 16S rRNA gene sequence analysis indicated that strain 169^T was most closely related to *Methanobacterium subterraneum* DSM 11074^T (96.8% sequence similarity) and *Methanobacterium formicicum* DSM 1535^T (96.4%). On the basis of its morphological, physiological and phylogenetic characteristics, strain 169^T (=DSM 22026^T=JCM 15797^T) represents a novel species of the genus *Methanobacterium*, for which the name *Methanobacterium kanagiense* sp. nov. is proposed.

Methane is one of the greenhouse gases and flooded rice fields are estimated to be a significant source of atmospheric methane (Prinn, 1994; Cicerone & Oremland, 1988; Galchenko *et al.*, 1989). Methane is produced by methanogenic archaea, which play an important role in anaerobic ecosystems, such as flooded rice field soils, by performing the final step in the anaerobic decomposition of organic matter. Methanogenic bacteria have been isolated from various natural anoxic environments (Boone *et al.*, 1993; Garcia, 1990; Mah & Smith, 1981). Many strains have been isolated from rice field soils, including members of the genera *Methanobacterium*, *Methanobrevibacter*, *Methanoculleus*, *Methanosarcina* and *Methanocella* (Asakawa *et al.*, 1993, 1995; Fetzer *et al.*, 1993; Großkopf *et al.*, 1998; Joulain *et al.*, 1998; Raimbault, 1981; Rajagopal *et al.*, 1988; Sakai *et al.*,

2008). In anoxic rice-field soils, hydrogen and acetate are the main methanogenic substrates (Conrad *et al.*, 1989a; Takai, 1970) and are produced as a result of fermentative metabolism or the activity of syntrophic associations between volatile fatty acid (acetate, propionate or butyrate)-oxidizing, hydrogen-producing bacteria and hydrogenotrophic methanogenic archaea (Dong & Stams, 1995; Schink, 1992; Stams, 1994). A high proportion (95%–97%) of the hydrogenotrophic methanogenesis in rice-field soils occurs in syntrophic associations (Conrad *et al.*, 1989b); however, the details of natural anoxic habitats remain, largely, to be determined. To understand the ecology of methanogenic archaea as hydrogen scavengers in anoxic environments, characterization and identification of methanogenic isolates with syntrophic associations are important. In this study, we describe the isolation and characterization of a methanogenic archaeon, designated strain 169^T, obtained from an anaerobic, propionate-oxidizing enrichment culture derived from rice-field soil as an inoculant. Strain 169^T, which uses only hydrogen and carbon dioxide as methanogenic substrates, is physiologically and phylogenetically distinct from other members of

Abbreviations: Pei, pseudomurein endoisopeptidase; rPeiW, recombinant pseudomurein endoisopeptidase.

The DDBJ/EMBL/GenBank accession numbers for the 16S rRNA and *mcrA* gene sequences of strain 169^T are AB368917 and AB551869, respectively.

the genus *Methanobacterium*. Therefore, it is proposed that strain 169^T represents a novel species of the genus *Methanobacterium*.

The sampling site was a rice-field plot at Kanagi Farm, part of the Teaching and Research Center for Bio-coexistence, University Farm, Faculty of Agriculture and Life Science, Hirosaki University. Sampling was performed in August 2002.

All procedures were conducted under anaerobic conditions. The basal medium used for routine cultivation was prepared as described previously (Widdel & Pfennig, 1981), except that sodium sulphate was omitted. It contained (l⁻¹): 0.2 g KH₂PO₄, 0.25 g NH₄Cl, 1.0 g NaCl, 0.4 g MgCl₂·6H₂O, 0.5 g KCl, 0.15 g CaCl₂·2H₂O, 1 ml trace element solution SL-7 (Widdel & Pfennig, 1981), 0.4 mg resazurin, 2.52 g NaHCO₃, 1 ml Na₂SeO₃·5H₂O (0.3 mg l⁻¹), 1 ml Na₂WO₄·2H₂O (0.4 mg l⁻¹), 2 ml *p*-aminobenzoic acid (40 mg l⁻¹), 2 ml D-(+)-biotin (10 mg l⁻¹), 2 ml thiamine chloride (100 mg l⁻¹) and 1 ml vitamin B₁₂ (50 mg l⁻¹). The pH was adjusted to 7.2–7.5 by addition of an anaerobic, sterile solution of 2 M HCl.

For enrichment of the propionate-degrading consortia present in the soil of a rice field, 5 g samples of soil were inoculated into 100 ml of basal medium in 125 ml serum bottles containing 20 mM sodium propionate followed by static incubation at 30 °C in an oxygen-free nitrogen atmosphere. After the complete consumption of propionate and acetate, which transiently accumulated as an intermediate during the methanogenic degradation of propionate, 1 ml of the culture, including sediment, was transferred into fresh medium and incubated as before. After seven transfers using the same medium, a hydrogenotrophic methanogen was isolated by repeated application of Hungate's roll tube technique (Hungate, 1969) using hydrogen/carbon dioxide (80:20, 2 atm) as a methanogenic substrate, which was also used for subsequent cultivation of the isolate, unless stated otherwise.

Levels of propionate and acetate were determined by GC (Shimadzu model GC-8A) using a Porapak N column (Waters) connected to a flame-ionization detector. The oven temperature was 180 °C and nitrogen was used as the carrier gas. Levels of methane and hydrogen were determined by GC using a WG-100 column (GL Science) connected to a thermal conductivity detector. The oven temperature was 60 °C and argon was used as the carrier gas.

Phase-contrast and epifluorescence microscopy were carried out with an Olympus model BX50 photomicroscope. Autofluorescence of the cells was observed with a V (BP-400-410) excitation filter. Gram staining was carried out according to the method of Bartholomew & Mittwer (1952). For electron microscopy, cells were collected from late-exponential-phase cultures by centrifugation at 7000 g for 10 min at 4 °C and washed twice in sterilized double-distilled water. For transmission electron microscopy, cells

were resuspended in double-distilled water and negatively stained with 0.5 % phosphotungstic acid (pH 7). For ultrathin sections, cells were fixed in 2.5 % glutaraldehyde for 18 h and post-fixed in 1 % osmium tetroxide for 1.5 h in Kellenberger buffer (pH 6) (Kellenberger *et al.*, 1958) at 4 °C. The fixed cells were embedded in Spurr's resin and sectioned with glass knives on an LKB 2188-NOVA ultramicrotome. Sections were stained with uranyl acetate and post-stained with lead citrate. All of the specimens for transmission electron microscopy were examined with a JEOL model JEM-2000 EX electron microscope operated at 80 kV.

Genomic DNA of strain 169^T was extracted from pseudomurein endoisopeptidase (Pei)-treated cells according to the method of Nakamura *et al.* (2006). Pei from *Methanothermobacter wolfeii* was kindly provided by Dr Nakamura (Gifu University, Japan) as a recombinant enzyme (rPeiW) expressed in *Escherichia coli* (0.5 mg rPeiW ml⁻¹ H buffer: 50 mM HEPES, pH 7.0; 5 mM dithiothreitol; 20.8 mM Na₂S). Late-exponential-phase cells of strain 169^T were harvested by centrifugation at 7000 r.p.m. for 10 min at 4 °C. The cells were washed twice with H buffer and resuspended in H buffer to give a cell density of approximately 10¹⁰ c.f.u. ml⁻¹. An aliquot of 1 ml of the cell suspension was transferred into a 5 ml vial under a stream of oxygen-free nitrogen gas and the vial was sealed with a butyl-rubber stopper and closed with an aluminium cap. The vial was incubated at 70 °C for 10 min in a reciprocating water bath at 150 r.p.m., followed by the addition of 0.1 ml rPeiW and an additional incubation period of 1 h at 70 °C under the same operating conditions. The genomic DNA was extracted and purified by phenol/chloroform extraction followed by RNaseA treatment, a second phenol/chloroform extraction and ethanol precipitation. The dried DNA pellet was then resuspended in sterile water. Aliquots of 10 µl of the purified DNA (1 µg µl⁻¹) were hydrolysed with 0.02 units of P1 nuclease (Yamasa Shoyu) to yield mononucleotides. The DNA G + C content was determined by reversed-phase HPLC using a Shimadzu model LC-10ADVP system. Separation of the mononucleotides was performed at 40 °C and at a flow rate of 1.5 ml min⁻¹ using a YMC-Pack ODS-AQ column (150 × 6.0 mm, 5 µm particle size, 12 nm pore; YMC) and 10 mM phosphate buffer (pH 3.8) as the mobile phase. Each deoxyribonucleotide was detected by measuring A₂₇₀. An equimolar mixture of four deoxynucleotides was used as a control.

The following substrates were tested for utilization (20 mM unless stated): H₂/CO₂ (2 atm; 80:20, v/v), methanol, formate, acetate, methylamine, dimethylamine, trimethylamine, ethanol, 1-propanol, 2-propanol, 1-butanol, 2-butanol, 3-methyl-1-butanol, pyruvate, 1-pentanol, 2-pentanol and cyclopentanol. Substrate utilization was determined by the measurement of methane levels and the observation of the growth in comparison with a blank control.

Growth of strain 169^T was determined at 4–60 °C and in 0–15 % (w/v) NaCl. Salinity and pH tests were performed at 40 °C. As no growth was observed at NaCl concentrations above 8 %, and growth at 7 % was too slow to calculate over the test period of 4 weeks, the optimum concentration for growth was determined in 0, 0.1, 0.25, 0.5, 1, 3, and 5 % (w/v) NaCl. Media with different pH values were prepared by varying the concentration of NaHCO₃ or Na₂CO₃ and the pH was adjusted using sterile 5 % Na₂CO₃ or 2 M HCl solution. Growth at different pH values, temperatures and NaCl concentrations was monitored by measuring OD₄₂₀ over time using a photoelectric colorimeter (Mini Photo 10; Sanshin). The requirement of vitamins for growth was tested by transferring cultures into vitamin-depleted media containing possible growth factors and comparing these with control cultures that contained vitamins or did not contain any vitamins. Methane production was monitored during incubation at 40 °C for 10 days and evaluated by comparison to the controls.

All physiological experiments were performed in triplicate except for substrate utilization, salinity and vitamin requirement tests, which were performed in duplicate.

The effects of antibiotics on growth were determined using the following compounds: ampicillin, chloramphenicol, streptomycin, nalidixic acid, neomycin, bacitracin, vancomycin and penicillin. The susceptibility of strain 169^T to these antibiotics was determined using media containing one of the above antibiotics at concentrations of 100, 500, 1000 and 2000 mg l⁻¹. Duplicate cultures were incubated for 2 weeks and the effects of the antibiotics were determined by comparing the growth rates with control cultures containing no antibiotics. Growth rate was determined by monitoring OD₄₂₀.

Primers ArckF (5'-TTGATCCTGSCGGAGGCYACYGCT-3') and ArckR (5'-CCAGCCGCAGRTTCCCCTACGGC-3') (Oyaizu & Hiraishi, 1999) were used to amplify the partial 16S rRNA gene of strain 169^T. Aliquots (1 µl) of cell suspension were added to a mixture of 1.25 U *Taq* DNA polymerase (HotStarTaq; Qiagen), 10 × PCR buffer (with 15 mM MgCl₂; Qiagen), 0.2 mM dNTP, the two primers (0.5 µM) and double-distilled water to a final volume of 50 µl. The thermal program included an initial denaturation step (95 °C, 15 min) followed by 35 cycles of denaturation (95 °C, 60 s), annealing (55 °C, 60 s) and extension (72 °C, 90 s) with a final extension step (72 °C for 6 min). The amplified products were cloned into pUC19 and sequenced with a GeneRapid DNA Sequencer (Amersham Pharmacia Biotech). A partial fragment of the *mcrA* gene of strain 169^T was amplified using the primers *mcrA*-f (5'-GGTGGTGTGCGATTACACARTAYGCW-ACAGC-3') and *mcrA*-r (5'-TTCATTGCRTAGTTWG-GRTAGTT-3') (Luton *et al.*, 2002). The reaction mixture consisted of 10 µl GoTaq Green Master Mix (Promega), each primer at 1 µM, 1 µl of the cell suspension as template and sterilized water to a final volume of 20 µl. PCR was performed with a denaturation step at 95 °C for 2 min

followed by 35 cycles of 95 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min with a final extension step of 72 °C for 5 min. The amplified products were cloned into pUC19 and sequenced with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The partial 16S rRNA gene sequence (1465 bp), the partial *mcrA* gene sequence (470 bp) and the deduced McrA amino acid sequence (156 aa) were compared with those in the GenBank database using the BLAST program. Sequences of the novel isolate and those of closely related members retrieved from the database were aligned using CLUSTAL W (Thompson *et al.*, 1994) and phylogenetic analysis was conducted using MEGA 4.0 software (Tamura *et al.*, 2007). Phylogenetic trees based on 16S rRNA gene and deduced McrA amino acid sequences were constructed using the neighbour-joining method (Saitou & Nei, 1987). Distances were computed using the maximum-composite-likelihood method (Tamura *et al.*, 2004). For both phylogenetic trees, bootstrap values (Felsenstein, 1985) were obtained from 1000 random resamplings of the data.

Strain 169^T was isolated from a propionate-oxidizing enrichment culture, originally obtained as an inoculant from rice-field soil. In this culture, large cell aggregates (~100 µm) consisted of autofluorescent methanogen-like cells; non-fluorescent cells were also frequently seen under the microscope. The autofluorescent cells were non-motile and straight or slightly curved rods with rounded ends, similar to other members of the genus *Methanobacterium*. An axenic culture of the apparently dominant methanogen was obtained by repeated application of the roll tube isolation procedure. The purity of the culture was examined in medium containing either 20 mM sodium sulphate and 20 mM lactate, 0.2 % glucose and 0.1 % polypeptone or 0.1 % yeast extract. No growth was observed and no contaminants were detected microscopically, indicating a pure culture.

Cells of strain 169^T were non-motile, Gram-reaction-variable, rod-shaped (1.6–5.0 × 0.35–0.5 µm) and usually occurred as single cells but sometimes formed long chains up to 30 µm. Negative staining preparations showed some fimbriae (5 nm in diameter) were present at both ends of the cell but no flagella were observed (Fig. 1a). The maximum number of fimbriae was ~10 per cell. Dark patches were seen distributed over the cell surface (Fig. 1b), similar to those seen in *Methanobacterium formicicum* DSM 1535^T; these represented intracytoplasmic membranous elements of unknown function (Langenberg *et al.*, 1968). The fibrillar meshwork previously observed with *M. formicicum* strain MF (Langenberg *et al.*, 1968) was not detected. Ultrathin sections showed a single-layered electron-dense cell wall about 6 nm thick, which is typical of Gram-positive bacteria (Fig. 1c). Strain 169^T showed fluorescence under UV light (420 nm), typical of methanogens. Cells of strain 169^T did not lyse in double-distilled water or 2 % (w/v) SDS after 90 min. Colonies in deep agar medium were circular, white-cream in colour and 0.5 mm in diameter after 3 months of incubation.

Fig. 1. Electron micrographs showing negatively stained cells of strain 169^T (a, b) and a transmission electron micrograph of an ultrathin section of strain 169^T (c). Bar, 200 nm.

Growth and methane production were observed at 15–45 °C and the optimum temperature for growth was 40 °C (Fig. 2a). No growth was observed at 4 °C and 50 °C. The pH range for growth was 6.5–9.6 (optimum pH 7.5–8.5) (Fig. 2b). No growth was observed below pH 6.0 or above pH 9.9. The salinity range for growth was 0–70 g NaCl l⁻¹ (0–1.2 M) and optimum growth was observed at 5 g NaCl l⁻¹ (0.086 M) (Fig. 2c). Growth and methane production were completely inhibited at 80 g NaCl l⁻¹ (1.37 M). Doubling time was 21 h under optimal conditions (pH 8.5, 40 °C). Strain 169^T grew only on hydrogen/carbon dioxide as sole carbon and energy sources. No growth factors or vitamins were required for growth. Addition of glucose (0.1 %), acetate (2 mM), tryptone (0.1 %), polypeptone (0.1 %), Casamino acids (0.1 %), cysteine-hydrochloride (0.025 %), trypticase peptone (0.1 %) and yeast extract (0.1 %) did not stimulate methane production. Methane production was reduced in medium containing 20 mM sodium formate (about 35 % of that produced by the control). The DNA G+C content of strain 169^T was 39.3 mol%.

Fig. 2. Growth rates of strain 169^T under different temperatures (a), pH levels (b) and NaCl concentrations (c). The effect of pH on growth (b) was tested under an atmosphere of H₂/CO₂ (90:10, v/v) at atmospheric pressure. As strain 169^T grew very slowly at around 7 % NaCl (c), the specific growth rate could not be calculated over the test period of 4 weeks. Specific growth rates are plotted. Data points represent the means of triplicate experiments and error bars represent SE.

Growth of strain 169^T was not inhibited by (mg l⁻¹) ampicillin (2000), vancomycin (2000) or penicillin (1000) but the addition of penicillin (2000), nalidixic acid (100)

Fig. 3. Phylogenetic tree based on 16S rRNA gene sequences, indicating the relationship of strain 169^T to other methanogenic archaea. The tree was constructed using the neighbour-joining method. *Methanothermobacter thermoautotrophicus* DSM 1053^T was used as an outgroup. Bootstrap values >50 %, based on 1000 resamplings, are shown at branch points. Accession numbers are given in parentheses. Bar, 0.01 substitutions per nucleotide position.

Fig. 4. Phylogenetic tree based on deduced McrA amino acid sequences, indicating the relationships between strain 169^T and other methanogenic archaea. The tree was reconstructed based on a distance matrix of 147 amino acid positions using PAM distance correction. *Methanopyrus kandleri* DSM 6324^T was used as an outgroup. Bootstrap values >50 %, based on 1000 resamplings, are shown at branch points. Accession numbers are given in parentheses. Bar, 0.05 substitutions per nucleotide position.

and streptomycin (100) decreased growth rate to 61.0 %, 53.8 % and 52.1 %, respectively, when compared to the control. These antibiotics caused greater reductions in growth rates at higher concentrations. Growth of strain 169^T was also inhibited by the addition of (mg l⁻¹) nalidixic acid (1000), neomycin (100), bacitracin (100) and chloramphenicol (100). As an archaeon, strain 169^T should not be susceptible to certain antibiotics effective against bacteria (streptomycin, nalidixic acid, neomycin, bacitracin and chloramphenicol). However, *Methanobacterium subterraneum* DSM 11074^T was also sensitive to chloramphenicol (40) and bacitracin (40) (Kotelnikova *et al.*, 1998) but not nalidixic acid (100) or streptomycin (100) (Kotelnikova *et al.*, 1998).

The almost full-length 16S rRNA gene sequence of strain 169^T (1465 bp) was amplified and sequenced. A phylogenetic tree was constructed based on the sequences of the isolate and those of closely related species (Fig. 3). The phylogenetic tree showed that a mesophilic group of

species of the genus *Methanobacterium* were the most closely related species to strain 169^T. The highest levels of 16S rRNA gene sequence similarity to strain 169^T were observed with *M. subterraneum* DSM 11074^T (96.8 %) and *Methanobacterium formicicum* DSM 1535^T (96.4 %). The partial sequence (470 bp) of the *mcrA* gene of strain 169^T was also determined and a phylogenetic tree based on the deduced amino acid sequence of *mcrA* genes was constructed (Fig. 4). This tree also supported the position of strain 169^T within the genus *Methanobacterium*. The closest relatives to strain 169^T based on the *mcrA* gene and deduced amino acid sequences were *M. subterraneum* DSM 11074^T (93.3 % amino acid sequence identity, 86.6 % nucleic acid sequence similarity) and *Methanobacterium aarhusense* DSM 15219^T (93.3 % amino acid sequence identity, 83.5 % nucleic acid sequence similarity).

16S rRNA gene sequence analysis showed that strain 169^T was related to known species of the genus *Methanobacterium* (Fig. 3). Based on its phenotypic

Table 1. Morphological and physiological characteristics of strain 169^T and closely related species of the genus *Methanobacterium*

Taxa: 1, strain 169^T (data from this study); 2, *M. formicicum* DSM 1535^T (Bryant & Boone, 1987); 3, *M. subterraneum* DSM 11074^T (Kotelnikova *et al.*, 1998); 4, *M. alcaliphilum* DSM 3387^T (Worakit *et al.*, 1986); 5, *M. palustre* DSM 3108^T (Zellner *et al.*, 1989). All strains utilize hydrogen/carbon dioxide as sources of energy and carbon. –, Negative; +, positive; v, variable; A, acetate; YE, yeast extract; P, peptone; ND, not determined.

Characteristic	1	2	3	4	5
Size (µm)	0.35–0.5 × 1.6–5	0.4–0.8 × 2–15	0.1–0.15 × 0.6–1.2	0.5–0.6 × 2–25	0.5 × 2.5–5
Long rods	+	+	–	+	+
Filaments	+	+	–	+	+
Fimbriae	+	+	ND	ND	ND
Gram reaction	v	v	+	–	+
Formate utilization	–	+	+	–	+
Chemoautotrophic	+	+	+	–	+
Growth stimulation by other compounds	–	A	–	YE, P	ND
pH range for growth (optimum)	6.5–9.6 (7.5–8.5)	6.6–7.8 (ND)	6.5–9.2 (7.8–8.8)	7.0–9.9 (8.1–9.1)	ND (7)
Temperature range for growth (°C) (optimum)	15–45 (40)	25–50 (37–45)	3.6–45 (20–40)	ND (37)	20–45 (33–37)
Tolerance of NaCl (M) (optimum)	0–1.2 (0.086)	ND	0–1.4 (0.2–1.25)	ND	0–0.3 (0.2)
DNA G + C content (mol%)	39.3	41–42	54.5	57	34

characteristics, strain 169^T could be distinguished from phylogenetically related taxa, as shown in Table 1. Closely related species *M. formicicum* and *M. subterraneum* used formate as a methanogenic substrate, whereas strain 169^T grew only on hydrogen/carbon dioxide as sole energy and carbon sources. Growth of *M. formicicum* was stimulated by acetate but growth of strain 169^T was not. The DNA G+C content of strain 169^T was 39.3 mol%, while that of *M. subterraneum* was 54.5 mol% G+C. Susceptibility to antibiotics (100 mg streptomycin l⁻¹ and 500 mg nalidixic acid l⁻¹) also differed between strain 169^T and *M. subterraneum*.

Based on its morphological, physiological and phylogenetic characteristics, strain 169^T represents a novel species of the genus *Methanobacterium*, for which the name *Methanobacterium kanagiense* sp. nov. is proposed.

Description of *Methanobacterium kanagiense* sp. nov.

Methanobacterium kanagiense (ka.na.gi.en'se. N.L. n. adj. *kanagiense* pertaining to Kanagi, Aomori, Japan, where the organism was isolated).

Cells are non-motile, Gram-reaction-variable and rod-shaped (1.6–5.0 × 0.35–0.5 µm). Hydrogen/carbon dioxide are the only substrates that support growth. Formate, methylamines, acetate, pyruvate, methanol and other alcohols plus carbon dioxide are not utilized. Grows autotrophically in mineral medium without any organic additives. Growth is not stimulated by acetate or yeast extract. Vitamins are not essential for growth. Grows at 15–45 °C, at pH 6.5–9.6, and with 0–70 g NaCl l⁻¹.

The type strain, 169^T (DSM 22026^T = JCM 15797^T), was isolated from an anaerobic, propionate-oxidizing enrichment culture derived from rice-field soil. The DNA G+C content of the type strain is 39.3 mol% (as determined by HPLC).

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