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Methylovulum psychrotolerans sp. nov., a cold-adapted methanotroph from low-temperature terrestrial environments and emended description of the genus Methylovulum

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Corresponding Author:	Svetlana N. Dedysh, Doctor of Sciences Winogradsky Institute of Microbiology, Russian Academy of Sciences Moscow, RUSSIAN FEDERATION
First Author:	Igor Y. Oshkin
Order of Authors:	Igor Y. Oshkin
	Svetlana E. Belova, PhD
	Olga V. Danilova, PhD
	Kirill K. Miroshnikov
	W. Irene C. Rijpstra
	Jaap S. Sinninghe Damste, Prof.
	Werner Liesack, Prof.
	Svetlana N. Dedysh, Doctor of Sciences
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Abstract:	Two isolates of aerobic methanotrophic bacteria, strains Sph1T and Sph2, were obtained from cold methane seeps in a floodplain of the river Mukhrinskaya, Irtysh basin, West Siberia. Another morphologically and phenotypically similar methanotroph, strain OZ2, was isolated from a sediment of a subarctic freshwater lake, Archangelsk region, Northern Russia. Cells of these three strains were Gram-stain-negative, light-pink-pigmented, non-motile, encapsulated, large cocci that contained an intracytoplasmic membrane system typical of type I methanotrophs. They possessed a particulate methane monooxygenase enzyme and utilized only methane and methanol. Strains Sph1T, Sph2, and OZ2 were able to grow at a pH range of 4.0-8.9 (optimum at 6.0-7.0) and at temperatures between 2 and 36°C. Although their temperature optimum was at 20-25°C, these methanotrophs grew well at lower temperatures, down to 4°C. The major cellular fatty acids were C16:1w5, C16:1w6, C16:1w7, C16:1w8, C16:0 and C14:0; the DNA G+C content was 51.4-51.9 mol%. Strains Sph1T, Sph2, and OZ2 displayed nearly identical (99.1-99.7% similarity) 16S rRNA gene sequences and belonged to the family Methylococcaceae of the class Gammaproteobacteria. The most closely related organism was Methylovulum miyakonense HT12T (96.0-96.5% 16S rRNA gene sequence similarity and 90% pmoA sequence identity). The novel isolates, however, differed from M. miyakonense HT12T by cell morphology, pigmentation, absence of soluble methane monooxygenase, more active growth at low temperatures, broader pH growth range, and higher DNA G+C content. Based on these differences, we propose a novel species, Methylovulum psychrotolerans sp. nov., for these methanotrophs. Strain Sph1T (=LMG 29227T =VKM B-3018T) is the type strain.

Methylovulum psychrotolerans sp. nov., a cold-adapted methanotroph from low-temperature terrestrial environments and emended description of the genus *Methylovulum*

Igor Y. Oshkin[1], Svetlana E. Belova[1], Olga V. Danilova[1], Kirill K. Miroshnikov, W. Irene C. Rijpstra[3], Jaap S. Sinninghe Damsté[3,4], Werner Liesack[5], Svetlana N. Dedysh[1]

[1]Winogradsky Institute of Microbiology, Research Center of Biotechnology of the Russian Academy of Sciences, Moscow 119071, Russia;

[2]M.V. Lomonosov Moscow State University, Moscow 119991, Russia;

[3]NIOZ Royal Netherlands Institute for Sea Research, Department of Marine Organic Biogeochemistry, PO Box 59, 1790 AB Den Burg, The Netherlands;

[4]Utrecht University, Faculty of Geosciences, Department of Earth Sciences, Geochemistry, Utrecht, the Netherlands;

[5]Max-Planck-Institut für terrestrische Mikrobiologie, D-35043 Marburg, Germany.

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Author for correspondence: Svetlana N. Dedysh

Tel: 7 (499) 135 0591. Fax: 7 (499) 135 6530. Email: dedysh@mail.ru

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences and the partial sequences of the *pmoA* gene of *Methylovulum psychrotolerans* strains Sph1^T, Sph2 and OZ2 are KT381578-KT381583, respectively.

ABSTRACT

Two isolates of aerobic methanotrophic bacteria, strains Sph1^T and Sph2, were obtained from cold methane seeps in a floodplain of the river Mukhrinskaya, Irtysh basin, West Siberia. Another morphologically and phenotypically similar methanotroph, strain OZ2, was isolated from a sediment of a subarctic freshwater lake, Archangelsk region, Northern Russia. Cells of these three strains were Gram-stain-negative, light-pink-pigmented, non-motile, encapsulated, large cocci that contained an intracytoplasmic membrane system typical of type I methanotrophs. They possessed a particulate methane monooxygenase enzyme and utilized only methane and methanol. Strains Sph1^T, Sph2, and OZ2 were able to grow at a pH range of 4.0-8.9 (optimum at 6.0-7.0) and at temperatures between 2 and 36°C. Although their temperature optimum was at 20-25°C, these methanotrophs grew well at lower temperatures, down to 4°C. The major cellular fatty acids were C16:1ω5, C16:1ω6, C16:1ω7, C16:1ω8, C16:0 and C14:0; the DNA G+C content was 51.4-51.9 mol%. Strains Sph1^T, Sph2, and OZ2 displayed nearly identical (99.1-99.7% similarity) 16S rRNA gene sequences and belonged to the family *Methylococcaceae* of the class *Gammaproteobacteria*. The most closely related organism was *Methylovulum miyakonense* HT12^T (96.0-96.5% 16S rRNA gene sequence similarity and 90% *pmoA* sequence identity). The novel isolates, however, differed from *M. miyakonense* HT12^T by cell morphology, pigmentation, absence of soluble methane monooxygenase, more active growth at low temperatures, broader pH growth range, and higher DNA G+C content. Based on these differences, we propose a novel species, *Methylovulum psychrotolerans* sp. nov., for these methanotrophs. Strain Sph1^T (=LMG 29227^T = VKM B-3018^T) is the type strain.

Keywords: *Methylovulum psychrotolerans* sp. nov., cold-adapted methanotrophs, methane oxidation at low temperatures, West Siberian methane seeps, subarctic freshwater lakes.

The genus *Methylovulum* belongs to the class *Gammaproteobacteria*, the family *Methylococcaceae*, and was so far represented by the only species, *Methylovulum miyakonense*, which accommodates strictly aerobic, neutrophilic, obligate utilizers of C1 compounds with type I intracytoplasmic membranes (ICM) and the ribulose-monophosphate pathway of carbon assimilation (Iguchi *et al*., 2011). The type strain of this species, *M. miyakonense* HT12^T, was isolated from a forest soil and was characterized as a mesophilic bacterium with the growth optimum at 24–32^{o}C. Several recent cultivation-independent studies, however, suggested that members of the genus *Methylovulum* are numerically abundant and metabolically active in low-temperature environments. Indeed, *Methylovulum*-like 16S rRNA gene sequences were detected using stable isotope probing technique in sediments from an arctic lake in northern Alaska (He *et al.*, 2012). These methanotrophs were also found in water discharged during summer seasons from Russell Glacier, a land-terminating outlet glacier at the western margin of the Greenland Ice Sheet (Dieser *et al*., 2014). In our recent study of cold methane seeps in floodplains of West Siberian rivers, *Methyluvulum*-related bacteria were also identified among the dominant methanotroph groups (Oshkin *et al.*, 2014). Our further efforts, therefore, were focused on obtaining cold-tolerant representatives of this genus in pure culture. Three strains of *Methylovulum*-like methanotrophs were obtained from two different permanently cold environments, i.e. West Siberian methane seeps and sediments of a subarctic freshwater lake. Here, we characterize these isolates and propose to classify them as belonging to a novel species of the genus *Methylovulum*.

Strains Sph1^T and Sph2 were isolated from mud suspensions sampled from two methane seeps located at a distance of 300 m from each other in the valley of the river Mukhrinskaya, Irtysh basin, West Siberia (60^o 53,358' N, 68^o 42,486' E). The seeps were characterized by low *in situ* temperatures (3.5 to 5 ^{o}C), high concentrations of emitted methane (70-99% of gases released from these bubbling pools) and near-neutral pH values of 6.8 to 6.9 (Oshkin *et al.*, 2014). Aliquots (0.5 ml) of mud suspensions were placed in 120 ml serum bottles containing 20 ml of

liquid diluted nitrate mineral salts medium (DNMS; Dunfield *et al.*, 2003) with the addition of 0.1% (by volume) of a trace elements stock solution containing (in grams per litre) EDTA, 5; $FeSO_4 \times 7H_2O$, 2; $ZnSO_4 \times 7H_2O$, 0.1; $MnCl_2 \times 4H_2O$, 0.03; $CoCl_2 \times 6H_2O$, 0.2; $CuCl_2 \times 5H_2O$, 0.1; $NiCl_2 \times 6H_2O$, 0.02, and Na_2MoO_4, 0.03. The medium pH was 6.8. The bottles were sealed with rubber septa, and CH_4 (30%, v/v) was added to the headspace using syringes equipped with disposable filters (0.22 µm). Bottles were incubated in static conditions at 9°C for 4 weeks until visible medium turbidity due to development of methanotrophic bacteria was observed. One of the major cell morphotypes in the resulting enrichment cultures was represented by large cocci, which could easily be recognised and traced in cultures by microscopic analysis. These cells became the main target of our further isolation efforts, which started with successive re-streaking of cell material from enrichment cultures on agar DNMS medium. Since development of large cocci was observed within a wide temperature range, the plates were further incubated at 20°C in desiccators under a methane/air (30:70) gas mixture. Colonies that appeared on the plates were picked randomly and examined microscopically in order to select for the target cell morphotype. Colonies composed mainly of large cocci were picked and transferred to the liquid medium MG2 with low salt content (in grams per litre) KH_2PO_4, 15; KNO_3, 15; $MgSO_4$, 15; NaCl, 20; $CaCl_2 \times 2H_2O$, 10; trace elements 0.1% (v/v). Multiple dilution series in this medium with CH_4 (30%, v/v) as the growth substrate resulted in isolation of the target methanotrophic bacteria represented by large cocci (Fig.1a), strains Sph1^T and Sph2, as pure cultures.

Another morphologically similar isolate, designated strain OZ2, was obtained from sediments of a subarctic, shallow (1.5-2 m depth), unnamed freshwater lake, Archangelsk region, Northern Russia (67^o 36,567' N, 53^o 35,317' E) using the same approach. This sampling site was also characterized by low *in situ* temperatures (5-7°C), and the sediment had a pH value of 6.5. The sample was collected from the surface layer (0-3 cm) of sediments in the littoral zone of this lake (depth 0.4 m at the sampling site).

In order to identify strains Sph1^T, Sph2 and OZ2 and to verify their relatedness, the 16S rRNA gene sequences of these bacteria were determined. PCR-mediated amplification of the 16S rRNA gene was performed using primers 9f (5'-GAGTTTGATCMTGGCTCAG-3') and 1492r (5'-ACGGYTACCTTGTTACGACTT-3') and reaction conditions described by Weisburg *et al.* (1991). Phylogenetic analysis was carried out using the ARB program package (Ludwig *et al*., 2004). The trees were constructed using distance-based (neighbor-joining), maximum-likelihood (DNAml), and maximum-parsimony methods. The significance levels of interior branch points obtained in neighbor-joining analysis were determined by bootstrap analysis (1000 data re-samplings) using PHYLIP (Felsenstein, 1989). The analysis revealed that strains Sph1^T, Sph2 and OZ2 possess nearly identical (99.1-99.7% similarity) 16S rRNA gene sequences and display 96.0-96.5% 16S rRNA gene similarity with *Methylovulum miyakonense* HT12^T, the neutrophilic and mesophilic methanotroph of the family *Methylococcaceae*, the class *Gammaproteobacteria* (Fig. 2). Among taxonomically uncharacterized organisms, the highest 16S rRNA gene sequence similarity (99% similarity) was observed with methanotrophic bacterium M200, which was isolated from a *Sphagnum* peat bog in the Netherlands (Kip *et al*., 2011). Since *M. miyakonense* was the closest taxonomically described relative of our isolates, the type strain of this species, DSM 23269^T, was used as a reference organism in our study. It was maintained on NMS medium (Whittenbury *et al*., 1970; DSMZ medium No 632), which was used for isolation and cultivation of *M. miyakonense* as described in the original publication (Iguchi *et al*., 2011). In all comparative tests, strains Sph1^T, Sph2, OZ2 and *Methylovulum miyakonense* DSM 23269^T were also grown on NMS medium under identical growth conditions.

For growth in liquid media, 120 ml serum bottles were used with a headspace/ liquid space ratio of 4:1. After inoculation, the bottles were sealed with silicone rubber septa, and methane was added aseptically using a syringe equipped with a disposable filter (0.22 µm) to achieve a 10-20% mixing ratio in the headspace. Bottles were incubated on a rotary shaker (100 rpm) at 20°C. Culture purity was verified by examination under phase-contrast and electron microscopy and by

plating on 10-fold diluted Luria–Bertani agar (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl). Only one cell morphotype was observed in cultures of strains Sph1^T, Sph2, OZ2, and no growth on diluted Luria–Bertani agar was observed after 3 weeks of incubation.

Morphological observations and cell-size measurements were made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss). Cells morphology was examined by using batch cultures grown to the early-exponential, late-exponential and stationary growth phases. Isolates Sph1^T, Sph2 and OZ2 were represented by Gram-negative and non-motile cocci (3-5 μм in diameter), which reproduced by binary fission and occurred singly or in pairs (Fig. 1a). Morphologically, they were clearly different from cells of *M. miyakonense* DSM 23269^T (Fig. 1b). Three-week-old colonies of strains Sph1^T, Sph2 and OZ2 were round, 2-3 mm in diameter, slimy, light-pink with an entire edge and a smooth surface. Liquid cultures displayed white to pale-pink turbidity. Formation of a surface pellicle in static liquid cultures was not observed.

For preparation of ultrathin sections, cells of the exponentially growing culture of strain Sph1^T were collected by centrifugation and pre-fixed with 1.5% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 6.5) for 1 h at 4^{o}C and then fixed with 1% (w/v) OsO_4 in the same buffer for 4 h at 20^{o}C. After dehydration in an ethanol series, the samples were embedded into Epon 812 epoxy resin. Thin sections were cut on an LKB-4800 microtome, stained with 3% (w/v) uranyl acetate in 70% (v/v) ethanol, and then were stained with lead citrate (Reynolds, 1963) at 20 °C for 4–5 min. The specimen samples were examined with a JEM-100B transmission electron microscope at an accelerating voltage of 80 kV. Examination of thin-sectioned cells of strain Sph1^T revealed a typical Gram-negative structure of the cell wall and the presence of intracytoplasmic membranes (ICM), arranged as stacks of vesicular disks (Fig. 1c) which is characteristic of type I methanotrophs.

Physiological tests were performed in liquid NMS medium with methane. Growth of strains Sph1^T, Sph2 and OZ2 was monitored by measuring OD_{600} for 2 weeks under a variety of

conditions, including temperatures of 2-37°C, pH 3.0-9.5 and NaCl concentrations of 0-5.0 % (w/v). Variations in the pH were achieved by mixing 0.1M solutions of H_3PO_4, KH_2PO_4, K_2HPO_4, and K_3PO_4. The utilization of potential carbon sources was examined using 0.1% (w/v) concentrations of the following compounds: methylamine, formate, glucose, sucrose, galactose, lactose, fructose, citrate, succinate, pyruvate, acetate, and tryptone. The ability to grow on methanol was tested in NMS medium containing 0.01–6% (v/v) methanol. The growth factor requirement was tested by supplementing NMS medium with 0.01% (w/v) Bacto tryptone or 0.001% (w/v) cyanocobalamin. Nitrogen sources were tested by replacing KNO_3 in liquid NMS medium with the following compounds at 0.05 % (w/v): ammonium chloride, sodium nitrate, urea, peptone, tryptone, yeast extract, Casamino acids, glycine, alanine, lysine, arginine, glutamate, glutamine, asparagine, tryptophan, methionine, threonine, histidine. For N_2-fixation experiments, a nitrate-free NMS medium was used. Growth was examined after 1 month of incubation.

Strains Sph1^T, Sph2 and OZ2 were able to grow only on methane and methanol. The specific growth rate on methane under optimal growth conditions was 0.08-0.09 h^{-1} for strain Sph1^T and 0.03-0.05 h^{-1} for strains Sph2 and OZ2. Growth factors were not required and also did not stimulate growth. By contrast, *M. miyakonense* DSM 23269^T grew better in the presence of growth factors, while it was also capable of growth in NMS medium without growth factors. Methanol supported growth of strains Sph1^T, Sph2 and OZ2 in the range of concentrations 0.1 – 5% (v/v); the best growth occurred at 0.7% (v/v). The specific growth rate on methanol was 0.024 h^{-1}. No growth was observed on multicarbon compounds. Nitrate, ammonium salts and casamino acids were used as sources of nitrogen. The novel isolates were also capable of slow growth (OD_{600} 0.15-0.20 after 3 weeks of incubation) in nitrogen-free medium under micro-oxic conditions (sealed flasks filled with liquid medium by ½ volume and with 30% air, 20% methane and 50% nitrogen in a headspace). The *nifH* (dinitrogenase reductase) gene, however, could not be detected in our isolates using the primers described by Poly *et al*. (2001), although the PCR

product of correct size was obtained in a positive control with DNA of *M. miyakonense* DSM 23269^T.

Strains Sph1^T, Sph2 and OZ2 grew in the pH range of 4.0 - 8.9, with the optimum at pH 6.0 - 7.0 (Suppl. Fig. S1). The temperature range for growth was 2-32 ºC for strains Sph1^T and OZ2 (Suppl. Fig. S2), and 2-36 ºC for strain Sph2. Although their temperature optimum was at 20-25°C, our isolates grew very well at lower temperatures, down to 4°C. Notably, the growth yield was always higher at 10°C (OD_{600} 1.8-2.0) than at 20°C (OD_{600} 1.2-1.5). As revealed in comparative tests, *M. miyakonense* DSM 23269^T was also able to grow at low temperatures, but its growth was less active than that of our isolates (Suppl. Fig. S3). Freshwater isolates Sph1^T, Sph2 and OZ2 were highly sensitive to salt stress; their growth was inhibited at NaCl concentrations above 0.1% (w/v).

For lipid analyses, strains Sph1^T, Sph2, OZ2 and *M. miyakonense* DSM 23269^T were grown in parallel, at 20°C, on liquid NMS medium with methane and harvested in the late exponential growth phase. Lipids were analyzed following the procedure described by Sinninghe Damsté *et al*. (2011). The obtained fatty acid profiles in cells of strains Sph1^T, Sph2 and OZ2 were highly similar to each other (Table 1) and were defined by the predominance of monounsaturated C16 fatty acids, which is typical for type I methanotrophs (Bowman *et al*., 1991; Bowman *et al*., 1993). The major fatty acids were C16:1ω5, C16:1ω6*c*. C16:1ω7, C16:1ω8, C16:0 and C14:0 fatty acids. Highly similar fatty acid composition was previously reported for closely related but taxonomically uncharacterized methanotroph, strain M200 (Kip *et al*., 2011). The double bond positions were determined by interpretation of the mass spectral fragmentation pattern of the DMDS (dimethyl disulfide) derivatives of the unsaturated fatty acids as described by Nicols *et al*. (1986). Notably, the fatty acid profile of *M. miyakonense* DSM 23269^T was similar to those in our isolates (Table 1). This was an unexpected finding because the original description of this species stated the absence of monounsaturated C16 fatty acids and listed C16:0 and C14:0 as two

major cellular fatty acids in *M. miyakonense* (Iguchi *et al*., 2011). In order to verify our data, we determined the partial (~700 bp) 16S rRNA gene sequence of strain obtained from DSMZ and confirmed its identity with the respective gene sequence of *M. miyakonense* HT12^T deposited in the GenBank under accession number AB501287. We then repeated cultivation, collected another batch of biomass of *M. miyakonense* DSM 23269^T and repeated fatty acid analysis. The latter confirmed the data shown in Table 1.

The DNA base composition of strains Sph1^T, Sph2 and OZ2 was determined by thermal denaturation using a Unicam SP1800 spectrophotometer (UK) at a heating rate of 0.5°C min^{-1}. The mol % G+C value was calculated according to Owen *et al.* (1969). The DNA of *Escherichia coli* K-12 was used as the standard. The DNA G+C content of our isolates was in the range of 51.4-51.9 mol%.

Partial fragments of the *pmoA* gene, which encodes the active-site polypeptide of particulate methane monooxygenase (pMMO), were amplified using the primers and the reaction conditions described by Holmes *et al*. (1995). Phylogenetic analysis based on fragments of the *pmoA* gene revealed that strains Sph1^T, Sph2, OZ2 display 90% nucleotide sequence identity (96.3% derived amino acid sequence identity) to *pmoA* gene fragments from *M. miyakonense* HT12^T (Fig. 3). The *mmoX* gene encoding a subunit of soluble MMO could not be amplified from DNA of our isolates with any of the previously described *mmoX*-targeted primers (Auman *et al*., 2000, McDonald *et al*., 2001, Miguez *et al*.. 1997, Hutchens *et al*., 2004). The colorimetric naphthalene oxidation test (Graham *et al.*, 1992) for sMMO activity in cells of strains Sph1^T, Sph2 and OZ2 grown on Cu-free NMS medium was also negative, although bright purple color developed on plates with sMMO-possessing M. *miyakonense* DSM 23269^T, which was used as a positive control in this test. The results suggest that sMMO is not present in any of the three novel isolates.

In summary, 16S rRNA and *pmoA* gene phylogenies as well as fatty acid profiles characterize strains Sph1^T, Sph2 and OZ2 as members of the genus *Methylovulum*. However, our novel isolates differed from the only so far described species of this genus, *M. miyakonense*, by cell morphology, pigmentation, absence of sMMO, more active growth at low temperatures, broader pH growth range and higher DNA G+C content (Table 2). Based on these differences, we propose to classify strains Sph1^T, Sph2 and OZ2 as belonging to a novel, cold-adapted species of the genus *Methylovulum*, *Methylovulum psychrotolerans* sp. nov.

Description of *Methylovulum psychrotolerans* sp. nov.

Methylovulum psychrotolerans (psy.chro.to′le.rans. Gr. adj. *psychros* – cold; L. pres. part. *tolerans* tolerant; N.L. part. adj. *psychrotolerans* – cold-tolerant).

Gram-negative, non-motile cocci, 3-5 μм in diameter. Cells occur singly or in pairs and are covered by large capsules. Possess stacks of intracytoplasmic membranes typical of type I methanotrophs. Colonies are slimy, light-pink with an entire edge and a smooth surface. Liquid cultures display white to pale-pink homogeneous turbidity; no surface pellicle is formed. The temperature range for growth is 2-36°C with the optimum at 20-25°C. Growth occurs between pH 4.0 and 8.9 with the optimum at pH 6.0-7.0. Methane and methanol are the only growth substrates. Methane is oxidized by pMMO; sMMO is absent. Methanol is utilized at concentrations 0.1-5.0% (v/v); optimal growth occurs at 0.7% (v/v) CH_3OH. Growth factors are not required. NaCl inhibits growth at concentrations above 0.1 %. The predominant fatty acids are C16:1ω5*c*, C16:1ω6*c*, C16:1ω8*c*, C16:1ω7*c*, C16:0 and C14:0. The DNA G+C content is 51.4-51.9 mol%. The type strain, Sp1^T (=LMG 29227^T = VKM B-3018^T), was isolated from the cold methane seep in West Siberia.

Emended description of the genus *Methylovulum* Iguchi *et al.* 2011

Cells are Gram-stain-negative, aerobic, non-motile, coccoid- or short-rod-shaped and possess stacks of intracytoplasmic membranes, typical of type I methanotrophs. No cysts are formed. Growth is observed on methane and methanol as sole carbon sources. Methane is oxidized by pMMO; the presence of sMMO is variable. C1 compounds are assimilated via the ribulose monophosphate pathway. Mesophilic and psychrotolerant. Growth of some species may be stimulated by growth factors. Major cellular fatty acids are C16: *ω5c*, *n*C16:1*ω6c*, C16:1*ω8c*, *n*C16:1*ω7c*, C16:0 and C14:0. The DNA G+C content is 50.7-51.9 mol%. Phylogenetically, a member of the family *Methylococcaceae*, in the class *Gammaproteobacteria*. The type species is *Methylovulum miyakonense*.

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Table 1. PLFA contents of strains Sph1^T, Sph2 and OZ2 in comparison to *M. miyakonense* DSM 23269^T. Major fatty acids (≥5% of total) are shown in bold. Values are percentages of total fatty acids. Strains: 1, Sph1^T; 2, Sph2; 3, OZ2; 4, *M. miyakonense* DSM 23269^T. All data are from this study.

Fatty acid	**1**	**2**	**3**	**4**
C14: 1*ω7c*	0.7	0.8	1.2	-
C14:0	**9.3**	**7.1**	**9.2**	**7.0**
C16:1*ω8c*	**25.3**	**30.1**	**22.7**	**19.2**
C16:1*ω7c*	**28.7**	**22.5**	**33.0**	**36.1**
C16:1*ω6c*	**6.2**	**6.4**	**5.7**	**6.1**
C16:1*ω5c*	**17.9**	**17.3**	**19.2**	**15.3**
C16:0	**6.3**	**11.4**	**6.2**	**12.1**
C18:1*ω9*	-	1.1	-	-
βOH-*n*C16:0	4.6	3.2	2.8	3.5

Table 2. Major characteristics that distinguish *Methylovulum psychrotolerans* sp. nov. from *Methylovulum miyakonense*. Species: 1, *Methylovulum psychrotolerans* sp. nov.; 2, *Methylovulum miyakonense.*

Characteristic	**1**	**2***
Cell shape	Cocci	Coccoids or short rods
Cell size (μm)	3 - 5	1.5-2.5×1.0-2.0
Color of colonies	Light-pink	Pale-brown
Presence of soluble MMO	-	+
Temperature range, °C (Temperature optimum, °C)	2 - 36 (20 – 25)	5-34 (24 – 32)
pH range (pH optimum)	4.0 - 8.9 (6.0 – 7.0)	6.0 - 7.5 (6.5)
G+C content (mol %)	51.3-51.9	50.7**

*Data are taken from Iguchi *et al*., 2011.

** Data are shown based on genome analysis (Hamilton *et al*., 2015).

FIGURE CAPTIONS

Fig. 1. Phase-contrast micrographs of cells of strains Sph1^T (**a**) and M. *miyakonense* DSM 23269^T (**b**) grown in liquid NMS medium under methane for 5 days; bar, 5 μm. **(c)** Electron micrograph of ultrathin section of a dividing cell of strain Sph1^T; bar, 1 μm. ICM, intracytoplasmic membranes.

Fig. 2. 16S rRNA gene-based neighbor-joining tree showing the phylogenetic position of strains Sph1^T, Sph2 and OZ2 in relation to other members of the family *Methylococcaceae*. Bootstrap values (percentages of 1000 data resamplings) >80% are shown. Black circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. The type II methanotrophs *Methyloferula stellata* AR4 (FR686343), *Methylocella silvestris* BL2 (AJ491847), *Methylocapsa acidiphila* B2 (AJ278726), *Methylosinus sporium* (Y18946), *Methylosinus trichosporium* OB3b (Y18947), and *Methylocystis parvus* (Y18945) were used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

Fig. 3. Unrooted neighbor-joining tree constructed based on 141 deduced amino acid sites of partial *pmoA* gene sequences, showing the position of strains Sph1^T, Sph2 and OZ2 relative to other type I and type II methanotrophs. Bootstrap values (percentages of 1000 data resamplings) >80% are shown. Black circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. Bar, 0.1 substitutions per amino acid position.

Figure 1

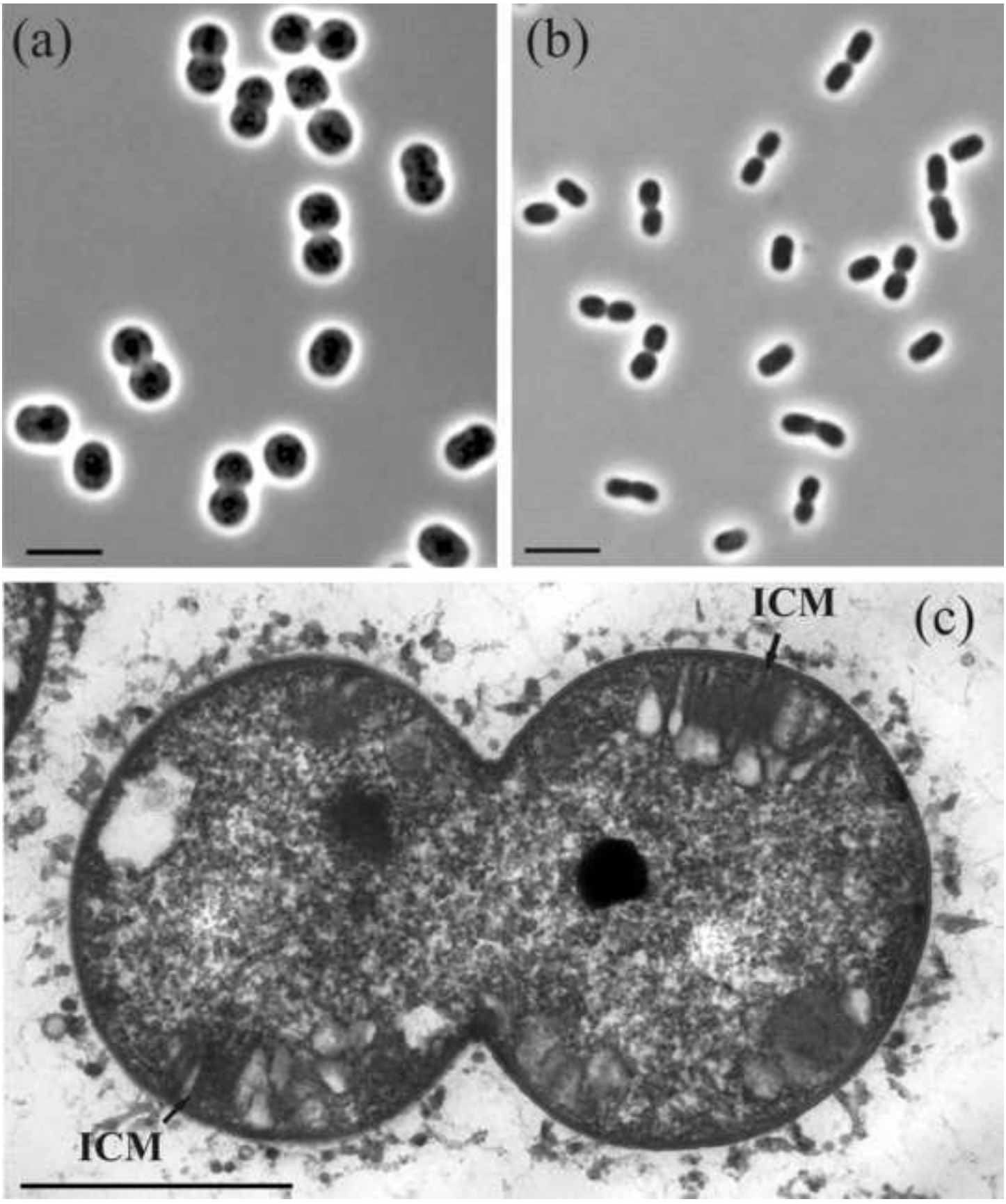


Figure 2

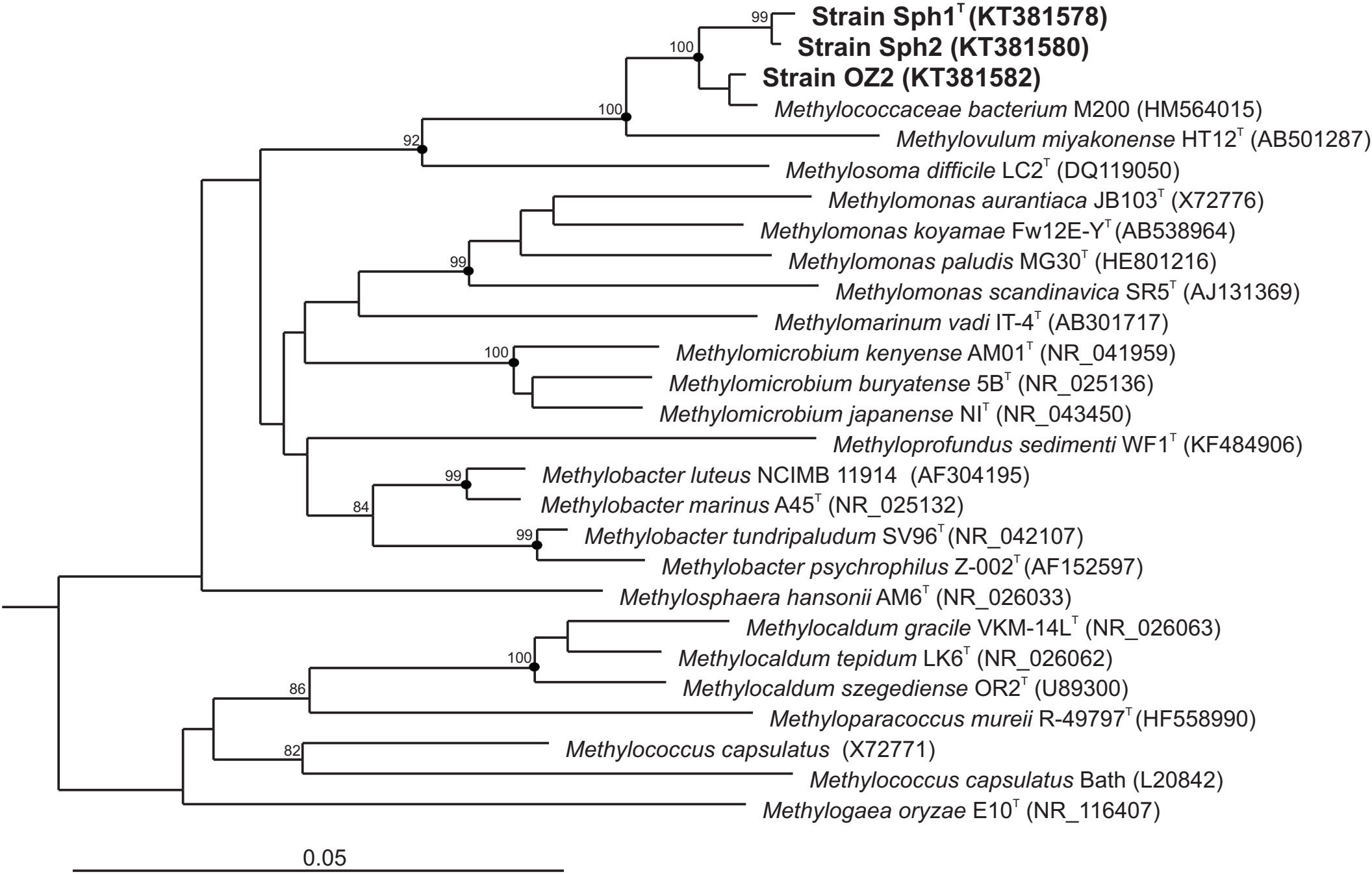
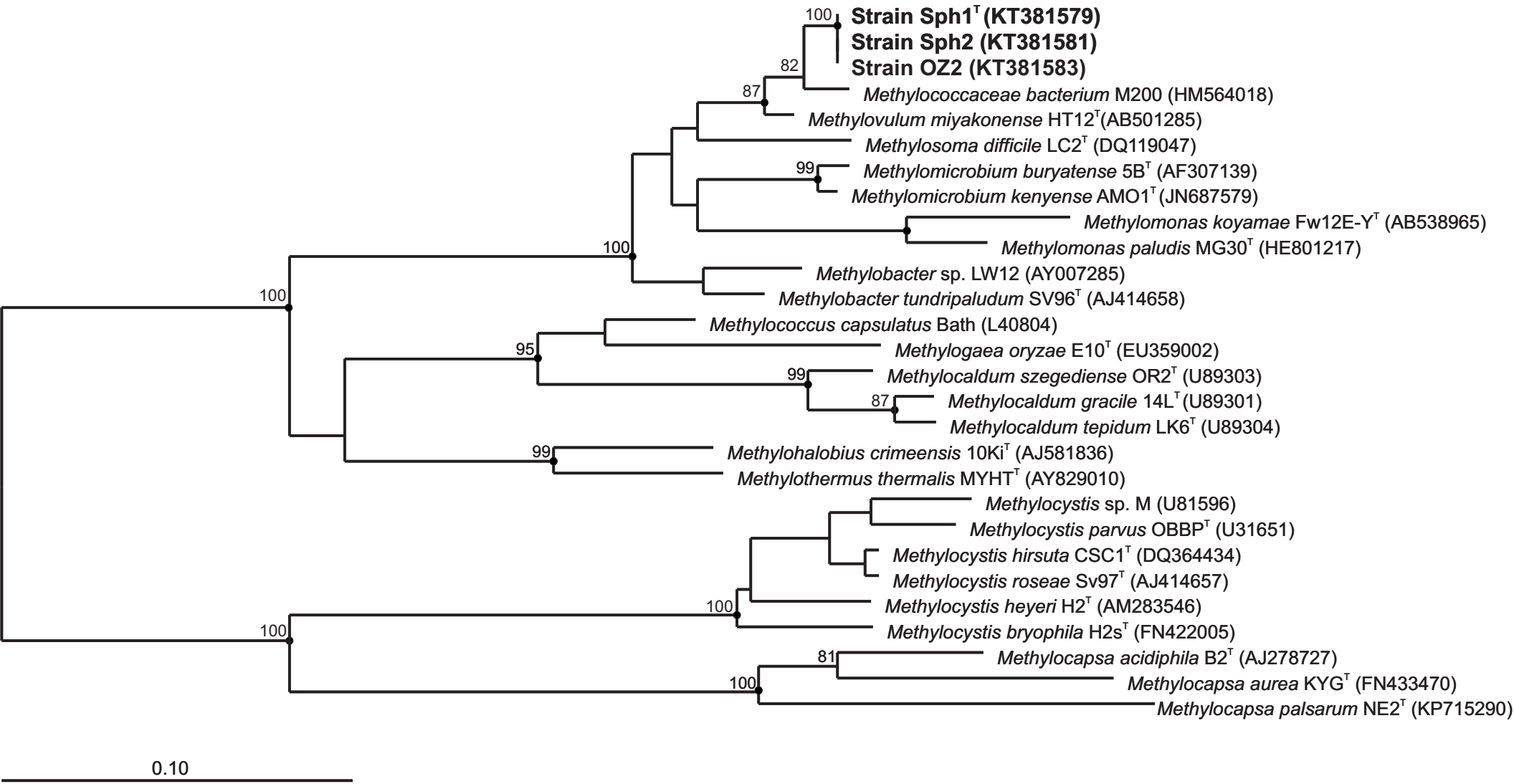
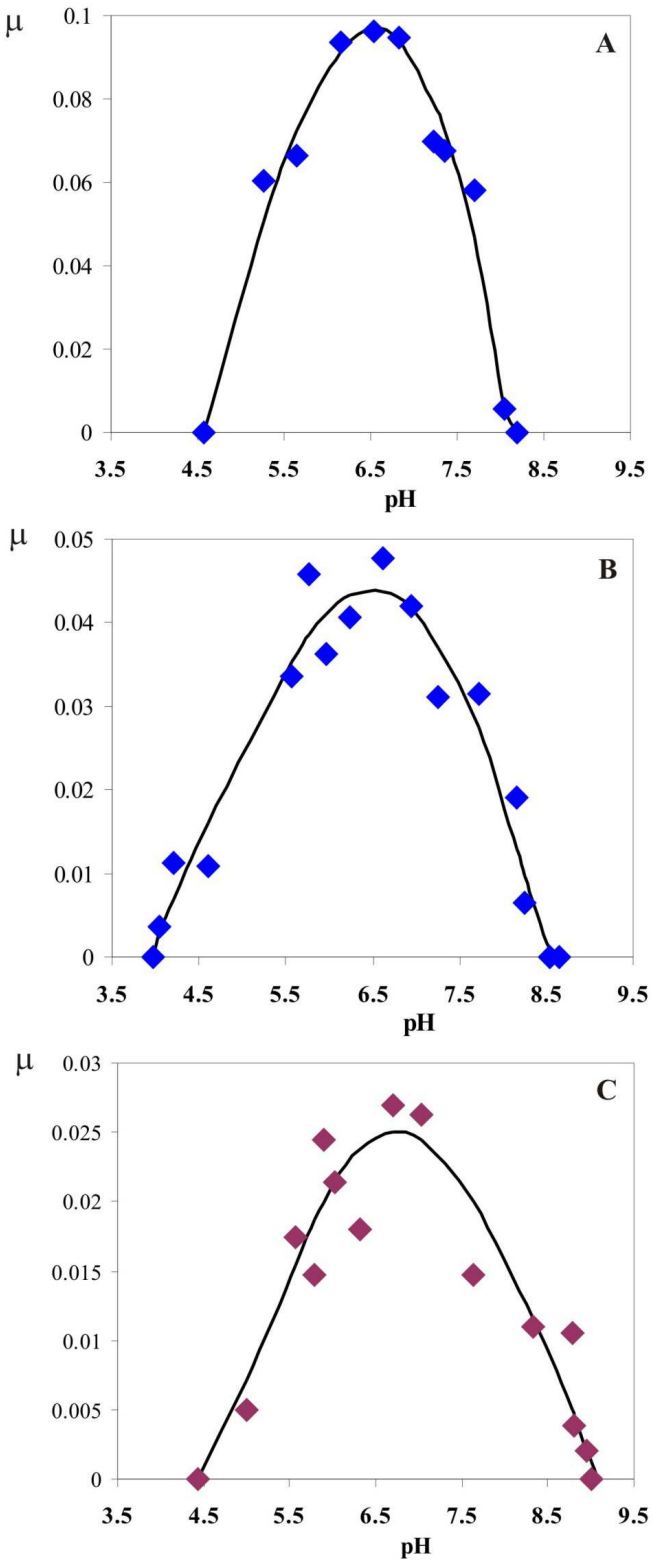
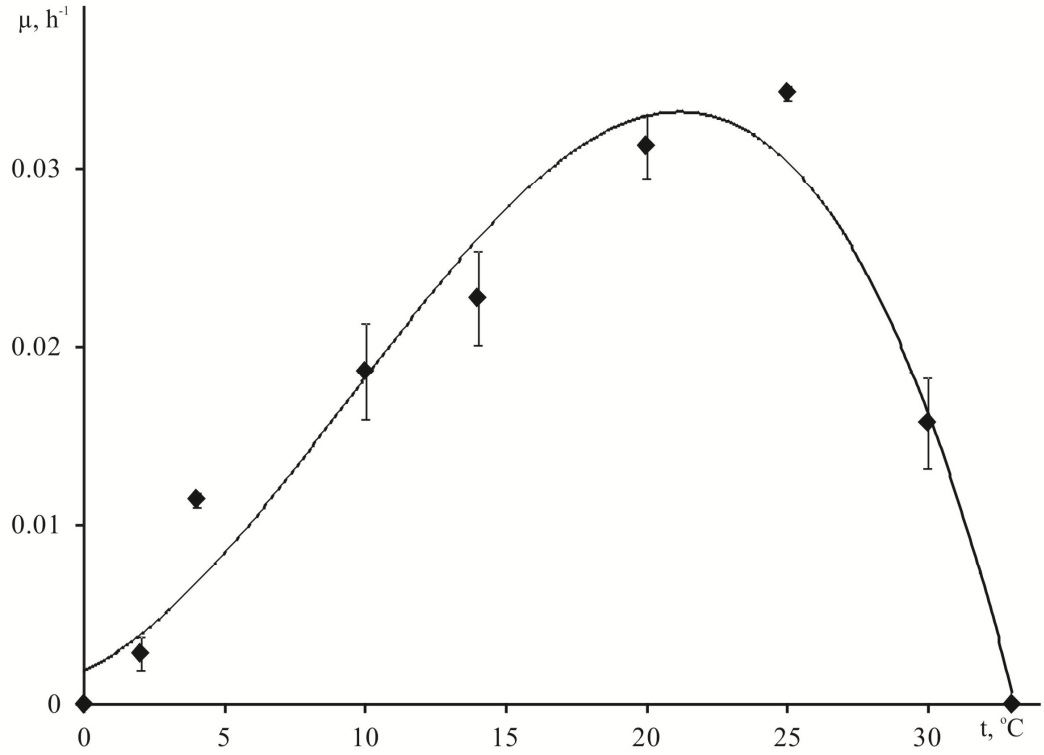


Figure 3

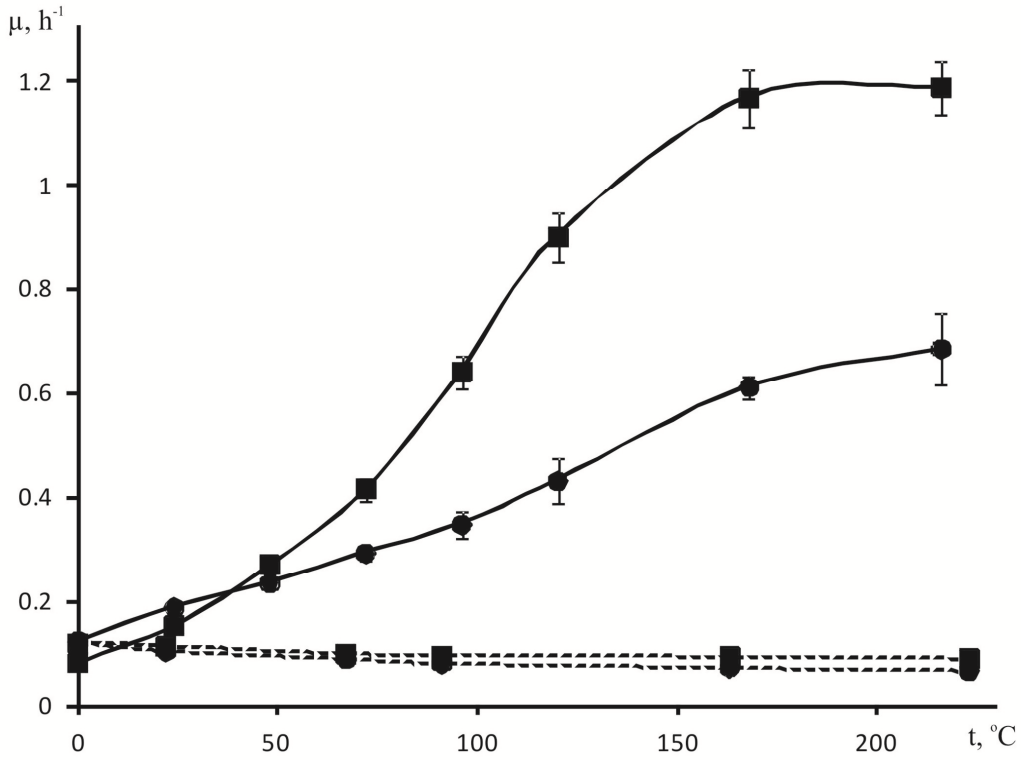




Supplementary Fig. S1. Influence of pH on the specific growth rate (μ) of strains Sph1^T(**A**), Sph2 (**B**) and OZ2 (**C**).



Supplementary Fig. S2. Influence of temperature on the specific growth rate (μ) of strain Sph1^T.



Supplementary Fig. S3. Growth dynamics of strain Sph1^T (squares) and *Methylovulum miyakonense* DSM 23269^T (circles) in NMS medium with methane at 10°C. To ensure optimal growth conditions, the medium for *Methylovulum miyakonense* DSM 23269^T was supplemented with growth factors as recommended by Iguchi *et al*., 2010. Dashed lines represent control incubations without methane. All incubations were made in triplicate. Where error bars are not seen, they are hidden behind symbols.

Reference: **Iguchi, H., Yurimoto, H. & Sakai, Y. (2011).** *Methylovulum miyakonense* gen. nov., sp. nov., a type I methanotroph isolated from forest soil. *Int J Syst Evol Microbiol* **61**, 810-815.