Methylocapsa palsarum sp. nov., a methanotroph isolated from a subArctic discontinuous permafrost ecosystem

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An aerobic methanotrophic bacterium was isolated from a collapsed palsal soil in northern Norway and designated strain NE2T. Cells of this strain were Gram-stain-negative, non-motile, non-pigmented, slightly curved thick rods that multiplied by normal cell division. The cells possessed a particulate methane monooxygenase enzyme (pMMO) and utilized methane and methanol. Strain NE2T grew in a wide pH range of 4.1–8.0 (optimum pH 5.2–6.5) at temperatures between 6 and 32 °C (optimum 18–25 °C), and was capable of atmospheric nitrogen fixation under reduced oxygen tension. The major cellular fatty acids were C18:1ω7c, C16:0 and C16:1ω7c, and the DNA G+C content was 61.7 mol%. The isolate belonged to the family Beijerinckiaecae of the class Alphaproteobacteria and was most closely related to the facultative methanotroph Methylocapsa aurea KYG1 (98.3 % 16S rRNA gene sequence similarity and 84 % PmoA sequence identity). However, strain NE2T differed from Methylocapsa aurea KYG1 by cell morphology, the absence of pigmentation, inability to grow on acetate, broader pH growth range, and higher tolerance to NaCl. Therefore, strain NE2T represents a novel species of the genus Methylocapsa, for which we propose the name Methylocapsa palsarum sp. nov. The type strain is NE2T (=LMG 28715T=VKM B-2945T).

The genus Methylocapsa was originally proposed to accommodate aerobic, mildly acidophilic, encapsulated, non-motile, dinitrogen-fixing methanotrophs that possess particulate methane monoxygenase (pMMO) (Dedys et al., 2002). Cells of these bacteria contain well-developed intracellular membranes (ICM) packed in parallel on one side of the cell. At the time of writing, this genus includes two species with validly published names, Methylocapsa acidiphila (Dedys et al., 2002) and Methylocapsa aurea (Dunfield et al., 2010). The type species of this genus, Methylocapsa acidiphila, is represented by obligate methanotrophs that utilize only methane and methanol as growth substrates and display a unique ability to grow actively in nitrogen-free media under fully aerobic conditions (Dedys et al., 2004). By contrast, members of the second described species of this genus, Methylocapsa aurea, are facultative methanotrophs, which, in addition to methane and methanol, are also capable of growth on acetate (Dunfield et al., 2010).

Representatives of the genus Methylocapsa are typical inhabitants of acidic wetlands and soils (Dedys et al., 2003; Dedys, 2009). Methylocapsa-related transcripts of pmoA and nifH genes encoding pMMO and dinitrogen reductase, respectively, were detected in a subarctic palsa peatland, suggesting that these methanotrophs are metabolically active in this ecosystem (Liebner & Svenning, 2013). Further cultivation studies resulted in isolation of a novel Methylocapsa-like isolate, designated strain NE2T. Here, we characterize this novel methanotroph and determine its taxonomic position.

Strain NE2T was isolated from a sample of the moss Sphagnum lindbergii collected in August 2011 in northern Norway (69° 41.116’ N 29° 11.752’ E), at the transition from the subArctic to the Arctic. The sampling site represented a stabilized successional stage of a previously collapsed palsa (Seppälä, 1986). Samples of the wet moss (pore water pH 4.6) were placed in 120 ml serum bottles, sealed with rubber septa, and methane (20 %, v/v) was

Abbreviations: ICM, intracellular membranes; pMMO, particulate methane monoxygenase; sMMO, soluble methane monoxygenase.

The GenBank/EMBL/DDBJ accession numbers for the nearly complete 16S rRNA gene sequence and the partial pmoA, mxaF and nifH sequences of strain NE2T are KP715289–KP715292, respectively.
added to the headspace using syringes equipped with disposable filters (0.22 µm). Bottles were incubated in static conditions at 23 °C under LED light (type NIL 130F; PHILIPS). After incubation for 1.5 months, one of the Sphagnum plants was transferred to a new 120 ml serum bottle containing 20 ml mildly acidic (pH 5.5) mineral medium M2 (Dedysh et al., 2002) and 20 % methane (v/v) in the headspace. Turbidity due to growth of methanotrophs was observed in the bottle after incubation for 3 weeks in the dark. Dilution series prepared from this enrichment culture were streaked on Whatman polycarbonate filters (Nucleopore Track-Etch membrane with pore size 0.2 µm). The filters were left floating on the surface of liquid medium M2 in Petri dishes and incubated in plastic containers with 20 % methane (v/v) in the headspace. Colonies that appeared on these filters after incubation for 2 months were successively restreaked and left floating on diluted nitrate mineral salts medium (DNMS; Dunfield et al., 2003). This procedure was repeated until colonies containing morphologically uniform cells were obtained. One of these colonies was used to inoculate a serum bottle with DNMS medium and 20 % methane (v/v) in the headspace. The resulting culture, strain NE2\(^T\), grew well both in M2 and DNMS media. Growth in the latter medium, however, was non-homogeneous due to formation of large cell aggregates and, therefore, strain NE2\(^T\) was further maintained in liquid M2 medium and transferred at 1-month intervals.

In order to identify this isolate, the 16S rRNA gene sequence of strain NE2\(^T\) was determined. PCR-mediated amplification of the 16S rRNA gene was performed using primers 9f and 1492r 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 reconstructed using distance-based (neighbour-joining), maximum-likelihood (DNAML), and maximum-parsimony methods. The significance levels of interior branch points obtained in neighbour-joining analysis were determined by bootstrap analysis (1000 data resamplings) using PHYLIP (Felsenstein, 1989). Comparative 16S rRNA gene sequence analysis revealed that strain NE2\(^T\) belongs to the family Beijerinckiaceae, the class Alphaproteobacteria, and is most closely related to the facultative methanotroph Methylocapsa aurea KYG\(^T\) (98.3 % 16S rRNA gene similarity) (Fig. 1). Therefore, Methylocapsa aurea KYG\(^T\) was used as a reference strain in our study.

The absence of heterotrophic satellites in strain NE2\(^T\) was checked by phase-contrast and electron microscopy and by plating onto 1 : 10 diluted R2A medium (Difco). Only one cell morphotype was observed under light microscopy and no growth on diluted R2A medium was observed after incubation for 3 weeks.

Morphological observations and cell size measurements were made with an Axioplan 2 microscope and Axiowision 4.2 software (Carl Zeiss). Cells of strain NE2\(^T\) were Gram-stain-negative, non-motile, encapsulated, slightly curved thick rods, 1.0–1.2 µm in width and 1.6–2.4 µm in length (Fig. 2a). Cells reproduced by binary fission and occurred singly or in irregularly shaped aggregates. The formation of rosettes was not observed. A distinctive bipolar appearance, which is characteristic of Methylocapsa aurea KYG\(^T\) (Fig. 2b) and occurs due to the presence of highly refractile intracellular granules of poly-β-hydroxybutyrate at each cell pole, was not observed for strain NE2\(^T\). On agar-solidified M2 medium, strain NE2\(^T\) formed small (1–2 mm in diameter), non-pigmented, round, slimy colonies. Liquid cultures displayed white turbidity. Incubation in static conditions often resulted in the formation of large cell aggregates.

For scanning electron microscopy, cells of an exponentially growing culture were fixed in 2.5 % glutaraldehyde in a freshly made growth medium, sedimented on poly-l-lysine coated glass, washed in PBS, post-fixed with 1 % aqueous osmium tetroxide, dehydrated in a graded series of ethanol and critical-point-dried in a Balzer Union CPD 020 critical point dryer. The specimen samples were mounted on aluminium stubs with silver glue, coated with gold/palladium in a Polaron Range Sputter Coater (Ringmer) and scanned with a Carl Zeiss Sigma Field Emission scanning electron microscope at the Electron Microscope Laboratory, UIT. The obtained image shows cells of strain NE2\(^T\) clamped to each other by means of a fibrous material (Fig. 1c). For preparation of ultrathin sections, cells of the exponentially growing cultures 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 °C and then fixed with 1 % (w/v) osmium tetroxide in the same buffer for 4 h at 20 °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. Thin-sectioned cells of strain NE2\(^T\) displayed well-developed intracellular membranes (ICM) packed in parallel on one side of the cell membrane (Fig. 2d). This type of ICM arrangement is typical for members of the genus Methylocapsa (Dedysh et al., 2002; Dunfield et al., 2010).

Physiological tests were carried out on cultures grown in liquid medium M2 with methane as the sole substrate. Growth of strain NE2\(^T\) was monitored by nephelometry at 410 nm using a 'Spectol' spectrophotometer (Carl Zeiss) for 2 weeks under a variety of conditions, including temperatures of 2–37 °C, pH 3.9–8.0, and NaCl concentrations of 0–3.0 % (w/v). Variations in the pH were achieved by mixing 0.1 M solutions of H\(_3\)PO\(_4\), K\(_2\)HPO\(_4\), and K\(_2\)HPO\(_4\). The following carbon sources (each at a concentration of 0.05 %, w/v) were examined to determine the range of substrates that could be utilized by strain NE2\(^T\): methanol, ethanol, formate, formic acid, glucose, fructose, arabinose, lactose, sucrose, maltose, galactose, acetate,
citrate, oxalate, malate, pyruvate and succinate. The capacity to utilize methanol at concentrations from 0.01 to 5 % (v/v) was determined in liquid M2 medium supplemented with methanol. Nitrogen sources were tested by replacing KNO₃ in medium M2 with 0.05 % (w/v) (NH₄)₂SO₄, NaNO₂, urea, hydroxylamine, peptone, L-serine, L-proline, L-alanine, L-asparagine and yeast extract.

For assessing N₂-fixation capability, a nitrate-free medium was used. In all substrate utilization tests, growth was examined after incubation for 1 month, and confirmed by comparison to a respective negative control.

Strain NE2ᵀ grew on methane or methanol as the sole carbon and energy sources.

Fig. 1. 16S rRNA gene-based neighbour-joining tree showing the phylogenetic position of strain NE2ᵀ in relation to other representatives of the family Beijerinckiaceae and some members of the family Methylocystaceae. Bootstrap values (1000 data resamplings) >50 % are shown at branch points. Filled circles indicate that the corresponding nodes were also recovered in the maximum-likelihood and maximum-parsimony trees. The type I methanotrophs Methylomonas methanica S1ᵀ (AF304196) and Methylococcus capsulatus Texasᵀ (NR_029241) were used as an outgroup (not shown). Bar, 0.05 substitutions per nucleotide position.
Fig. 2. (a, b) Phase-contrast micrographs of cells of strain NE2T (a) and *Methylocapsa aurea* KYG\(^T\) (b) grown in liquid medium M2 with methane for 7 days. (c) Scanning electron micrograph of cells of strain NE2T. (d) Electron micrograph of an ultrathin section of methane-grown cells of strain NE2T. Bars, 5 μm (a, b), 1 μm (c), 0.5 μm (d).
was 0.027 h⁻¹ (equivalent to a doubling time of 25 h). Methanol supported growth only when used at concentrations below 0.5 % (v/v); the most active growth occurred at 0.2 % (v/v). Growth factors were not required. In contrast to *Methylocapsa aurea KYG²*, strain NE2² was unable to grow on acetate as well as on other multi-carbon (Cₙ) compounds tested. Strain NE2² grew in a wide pH range of 4.1–8.0 with optimum growth at pH 5.2–6.5. This was dramatically different from *Methylocapsa aurea KYG¹*, which grew in a very narrow pH range of 5.2–7.2 and was unable to develop below pH 5.0. The temperature range for growth was 6–32 °C, with optimum growth at 18–25 °C. Strain NE2² did not require NaCl for growth and grew well in the presence of NaCl up to 0.1 % (w/v). Growth inhibition of 50 % was observed in the presence of 0.3 % (w/v) NaCl, and concentrations above 0.5 % (w/v) completely inhibited growth. Nitrogen sources included nitrates, urea, L-proline, L-alanine, L-asparagine, peptone and yeast extract (0.05 %, w/v). Surprisingly, strain NE2² did not utilize ammonium salts, which is different from both *Methylocapsa aurea KYG¹* and *Methylocapsa acidiphila B2²*. Strain NE2² was able to fix dinitrogen, although growth in nitrogen-free medium was more active under micro-oxic (sealed flasks filled with liquid medium by half-volume and with 50 % air and 50 % nitrogen in the headspace) than under fully oxic conditions. A partial fragment of the *nifH* gene (encoding dinitrogenase reductase) was amplified using primers and reaction conditions described by Dedysh *et al.* (2004). The *nifH* gene fragment from strain NE2² displayed highest similarity (89–90 % nucleotide sequence similarity and 95 % derived amino acid sequence identity) to the corresponding gene fragments from various strains of the genera *Bradyrhizobium* and *Azorhizobium*.

For cellular fatty acid analysis, strain NE2² was grown under the same growth conditions as described for *Methylocapsa aurea KYG¹* (Dunfield *et al.*, 2010), i.e., in batch cultures in DNMS medium at 24 °C for 10 days. The fatty acid profiles were analysed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described by Kämper & Kroppenstedt (1996). The cellular fatty acid profile of strain NE2² was similar to those of *Methylocapsa aurea KYG¹* and *Methylocapsa acidiphila B2²* (Table 1). The major cellular fatty acid in all of these methanotrophs was 11-*cis*-octadecenoic acid (C₁₈:1ω7c). However, the content of this fatty acid in strain NE2² (55 % of the total fatty acids), was lower than that in both described species of the genus *Methylocapsa* (78–82 %). In addition, the contents of C₁₆:1ω7c and C₁₆:0 fatty acids were significantly higher in strain NE2² than in *Methylocapsa aurea KYG¹* and *Methylocapsa acidiphila B2²*.

The DNA base composition of strain NE2² was determined by thermal denaturation using a Unicam SP1800 spectrophotometer at a heating rate of 0.5 °C min⁻¹. The mol% G+C value was calculated according to Owen *et al.* (1969) using *Escherichia coli* K-12 (G+C 51.7 mol%) as a standard. The DNA G+C content of strain NE2² was 61.7 mol%. This is within the range of values characteristic for other members the genus *Methylocapsa* (61.4–61.9 mol%) (Table 2).

Table 1. Cellular fatty acid composition of strain NE2² and other described members of the genus *Methylocapsa*

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tbody>
<tr>
<td>C₁₈:1ω7c</td>
<td>55.0</td>
<td>81.5</td>
<td>78.3</td>
</tr>
<tr>
<td>C₁₆:1ω7c</td>
<td>1.1</td>
<td>0.8</td>
<td>7.6</td>
</tr>
<tr>
<td>C₁₉:0</td>
<td>2.5</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C₁₉:0</td>
<td>0.4</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C₂₀:0</td>
<td>0.2</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

Partial fragments of the *pmoA* (active-site polypeptide of pMMO) and *mxaF* (large subunit of methanol dehydrogenase) genes were amplified from DNA of strain NE2² using the primers and the reaction conditions described by Holmes *et al.* (1995) and McDonald & Murrell (1997), respectively. Comparative analysis of the *pmoA* gene revealed strain NE2² belongs to the phylogenetic lineage defined by the genus *Methylocapsa* and displays only 79.8 % nucleotide sequence similarity (or 84 % derived amino acid sequence identity) to the *pmoA* gene fragment from *Methylocapsa aurea KYG¹* (Fig. 3). The *mxaF* gene fragment from strain NE2² was most closely related to *mxaF* from members of the genus *Methylocystis* (86–87 % nucleotide sequence similarity or 94–96 % derived amino acid sequence identity). Notably, the highest amino acid sequence identity (97–98 %) of MxaF from strain NE2² was observed with MxaF fragments retrieved from an acidic forest soil in the course of a study on active methylothrophs identified by means of stable isotope probing (Radajewski *et al.*, 2002). This suggests that strain NE2²-like methanotrophs might inhabit various acidic terrestrial environments. The *mmoX* gene encoding a subunit of...
soluble methane monooxygenase (sMMO) could not be amplified from DNA of strain NE2T with any of the previously described *mmoX*-targeted primers (McDonald et al., 1995; Miguez et al., 1997; Shigematsu et al., 1999; Auman et al., 2000; Vorobev et al., 2011). In order to confirm the absence of sMMO in strain NE2T, the colorimetric naphthalene oxidation test (Graham et al., 1992) was performed with *Methylocystis bryophila* H2sT as the positive control. As expected, this test was positive for sMMO-possessing *Methylocystis bryophila* H2sT, but negative for strain NE2T.

In summary, strain NE2T was phenotypically and genotypically distinct from the two currently described species of the genus *Methylocapsa* (Table 2). Phylogenetically, strain NE2T was most closely related to *Methylocapsa aurea* KYG1, but differed from it by cell morphology, the absence of pigmentation, inability to grow on acetate, broader pH growth range, and higher tolerance to NaCl. In addition, a DNA–DNA hybridization experiment was performed for strain NE2T and *Methylocapsa aurea* KYG1 as described by De Ley et al. (1970), and showed a DNA–DNA relatedness value of only 57% between these methanotrophs. Although displaying similar cell morphology, pH growth range and substrate utilization pattern, strain NE2T was phylogenetically distinct from *Methylocapsa acidiphila* B2T, and could be differentiated from this methanotroph by the inability to utilize ammonium salts as nitrogen sources and to grow in nitrogen-free media under fully oxic conditions. On the basis of these data, strain NE2T represents a novel species of the genus *Methylocapsa*, for which we propose the name *Methylocapsa palsarum* sp. nov.

### Description of *Methylocapsa palsarum* sp. nov.


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**Table 2.** Characteristics that differentiate strain NE2T and other described members of the genus *Methylocapsa*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>Cell morphology</td>
<td>Thick rods</td>
<td>Bipolar curved rods</td>
<td>Coccoids</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>None</td>
<td>Yellow</td>
<td>None</td>
</tr>
<tr>
<td>Growth on acetate</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>pH growth range</td>
<td>4.1–8.0</td>
<td>5.2–7.2</td>
<td>4.2–7.2</td>
</tr>
<tr>
<td>Optimal growth temperature (°C)</td>
<td>18–25</td>
<td>25–30</td>
<td>20–24</td>
</tr>
<tr>
<td>Sensitivity to NaCl (% w/v)</td>
<td>0.5</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Use of ammonium as nitrogen source</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Growth in nitrogen-free medium under fully oxic conditions</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Major fatty acids</td>
<td>C18 : 1ω7c, C16 : 1ω7c, C16 : 0</td>
<td>C18 : 1ω7c</td>
<td>C18 : 1ω7c</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>61.7</td>
<td>61.4</td>
<td>61.9*</td>
</tr>
</tbody>
</table>

*Data based on genome sequence analysis (Tamas et al., 2014).
The description is as for the genus but with the following additional traits. Cells are 1.0–1.2 μm wide and 1.6–2.4 μm long. Colony colour is white. Carbon sources include methane and methanol. Nitrogen sources are nitrates, urea, L-proline, L-alanine, L-asparagine, peptone and yeast extract. Fixes N2 via oxygen-sensitive dinitrogenase. Optimal growth occurs at 18–25 °C and pH 5.2–6.5. NaCl inhibits growth at a concentration of 0.5 % (w/v).

The type strain is NE2T (=LMG 28715T=VKM B-2945T), which was isolated from a collapsed palsar soil, northern Norway. The DNA G+C content of the type strain is 61.7 mol%.

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