Methyloferula stellata gen. nov., sp. nov., an acidophilic, obligately methanotrophic bacterium that possesses only a soluble methane monooxygenase

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Two strains of aerobic methanotrophic bacteria, AR4^T and SOP9, were isolated from acidic (pH 3.8–4.0) *Sphagnum* peat bogs in Russia. Another phenotypically similar isolate, strain LAY, was obtained from an acidic (pH 4.0) forest soil in Germany. Cells of these strains were Gram-negative, non-pigmented, non-motile, thin rods that multiplied by irregular cell division and formed rosettes or amorphous cell conglomerates. Similar to *Methylocella* species, strains AR4^T, SOP9 and LAY possessed only a soluble form of methane monooxygenase (sMMO) and lacked intracytoplasmic membranes. Growth occurred only on methane and methanol; the latter was the preferred growth substrate. mRNA transcripts of sMMO were detectable in cells when either methane or both methane and methanol were available. Carbon was assimilated via the serine and ribulose-bisphosphate (RuBP) pathways; nitrogen was fixed via an oxygen-sensitive nitrogenase. Strains AR4^T, SOP9 and LAY were moderately acidophilic, mesophilic organisms capable of growth between pH 3.5 and 7.2 (optimum pH 4.8–5.2) and at 4–33 °C (optimum 20–23 °C). The major cellular fatty acid was 18 : 1ω7c and the quinone was Q-10. The DNA G+C content was 55.6–57.5 mol%. The isolates belonged to the family *Beijerinckiaceae* of the class *Alphaproteobacteria* and were most closely related to the sMMO-possessing methanotrophs of the genus *Methylocella* (96.4–97.0 % 16S rRNA gene sequence similarity), particulate MMO (pMMO)-possessing methanotrophs of the genus *Methylocapsa* (96.1–97.0 %), facultative methylotrophs of the genus *Methylovirgula* (96.1–96.3 %) and non-methanotrophic organotrophs of the genus *Beijerinckia* (96.5–97.0 %). Phenotypically, strains AR4^T, SOP9 and LAY were most similar to *Methylocella* species, but differed from members of this genus by cell morphology, greater tolerance of low pH, detectable activities of RuBP pathway enzymes and inability to grow on multicarbon compounds. Therefore, we propose a novel genus and species, *Methyloferula stellata* gen. nov., sp. nov., to accommodate strains AR4^T, SOP9 and LAY. Strain AR4^T (=DSM 22108^T =LMG 25277^T =VKM B-2543^T) is the type strain of *Methyloferula stellata*.

Aerobic methanotrophs are a unique group of methylotrophic bacteria that utilize methane as a sole carbon and energy source. At present, methanotrophic capabilities are recognized in members of two bacterial phyla, the *Proteobacteria* and *Verrucomicrobia* (Hanson & Hanson, 1996; Op den Camp *et al.*, 2009). Almost all known

Abbreviations: ICM, intracytoplasmic membrane; PLFA, phospholipid fatty acid; pMMO, particulate-form methane monooxygenase; RuBP, ribulose 1,5-bisphosphate; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase; sMMO, soluble-form methane monooxygenase.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences and partial *mmoX* gene sequences of strains AR4^T, SOP9 and LAY are FR686343–FR686345 (16S rRNA gene) and FR686346–FR686348 (*mmoX*), respectively, and those for the partial *mxaF*, *nifH* and *rbcL* sequences of strain AR4^T are FR686349, FR686351 and FR686352, respectively.

Three supplementary figures are available with the online version of this paper.

methanotrophs possess a particulate methane monooxygenase enzyme (pMMO) and contain a well-developed intracytoplasmic membrane (ICM) system in which pMMO is bound. The only known exceptions are representatives of the genus *Methylocella*, which possess only a soluble methane monooxygenase (sMMO) and lack an extensive ICM system (Dedysh *et al.*, 2000, 2004a; Dunfield *et al.*, 2003; Chen *et al.*, 2010). Another unique characteristic of *Methylocella* species is the ability to utilize a number of multicarbon compounds (Dedysh *et al.*, 2005a; Dedysh & Dunfield, 2010). sMMO in *Methylocella* is repressed if an alternative, multicarbon growth substrate is present, but is not affected by copper ion availability (Theisen *et al.*, 2005).

Members of the genus *Methylocella* are widely distributed in acidic to neutral terrestrial environments, especially in peatlands and acidic soils (Dedysh & Dunfield, 2010). Due to the absence of pMMO in *Methylocella* species, these bacteria cannot be detected using a *pmoA*-based PCR assay considered universal and specific for all other known methanotrophs. However, they do possess the *mmoX* gene, encoding the α-subunit of sMMO, and can be detected via retrieval of these genes from the environment. Here, we describe three novel methanotrophs of a similar nature, strains AR4^T, SOP9 and LAY, which also lack a pMMO and use only an sMMO for methane oxidation. It is remarkable that the *mmoX* gene could not be amplified from DNA of our novel isolates with any of the previously known *mmoX*-targeted primers (McDonald *et al.*, 1995; Miguez *et al.*, 1997; Shigematsu *et al.*, 1999; Auman *et al.*, 2000). This explains why these methanotrophs escaped detection in all former cultivation-independent studies. In our paper, we present detailed characterization of these novel, sMMO-possessing methanotrophs and propose a novel genus and species for these bacteria.

Strains AR4^T and SOP9 were respectively isolated from an acidic peat soil (pH 3.8) sampled at a depth of 10 cm of the oligo-mesotrophic fen Torfjanoye, Archangelsk region, European North Russia (65° 01′ N 35° 44′ E), and a peat soil (pH 4.0) sampled at a depth of 10–20 cm of the *Sphagnum* peat bog Bakchar, West Siberia, Russia (56° 51′ N 82° 51′ E). Two methanotrophic enrichment cultures were obtained from these peat samples using liquid mineral medium MM2 of the following composition (per litre demineralized water): KH_2PO_4, 100 mg; $(NH_4)_2SO_4$, 100 mg; $MgSO_4.7H_2O$, 50 mg; $CaCl_2.2H_2O$, 20 mg; pH 4.8–5.2. Isolation of methanotrophs from the resulting enrichment cultures was achieved by plating an aliquot of the respective cell suspensions on medium MM2 solidified with gellan gum (Gel-Gro; ICN Biomedicals). The third isolate, strain LAY, was obtained from an acidic (pH 4.0) forest soil near Marburg, Germany, by plating a soil suspension onto the surface of diluted nitrate mineral agar salts (DNMS) medium at pH 5.8 as described by Dunfield *et al.* (2003). Inoculated plates were incubated for 1.5 months at 24 °C in a closed glass desiccator containing a headspace of 20 % (v/v) methane and 5 % CO_2 (v/v) in air. Colonies that developed on these plates were picked and purified by successive restreaking.

The isolates were maintained on their respective solid media (medium MM2 for strains AR4^T and SOP9 and DNMS medium for strain LAY) and in liquid cultures. For growth in liquid media, 500 ml screw-capped serum bottles were used with a headspace/liquid space ratio of 4 : 1. After inoculation, methanol was added aseptically to the cultures and the bottles were capped with silicone rubber septa to prevent loss of methanol by evaporation, or methane was added aseptically through silicone rubber septa to achieve a gas-mixing ratio in the headspace of approximately 10–20 %. Bottles were incubated on a rotary shaker (120 r.p.m.) at 24 °C.

Morphological observations and cell-size measurements were made with a Zeiss Axioplan 2 microscope and Axiovision 4.2 software (Zeiss). For the preparation of ultrathin sections, cells from cultures grown in liquid medium MM2 under methane were collected by centrifugation and pre-fixed with 1.5 % (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) for 1 h at 4 °C and then fixed in 1 % (w/v) OsO_4 in the same buffer for 4 h at 20 °C. After dehydration in an ethanol series, the samples were embedded in an Epon 812 epoxy resin. Thin sections were cut on an LKB-2128 Ultrotome, stained with 3 % (w/v) uranyl acetate in 70 % (v/v) ethanol and then post-stained with lead citrate (Reynolds, 1963) at 20 °C for 4–5 min. Samples were examined with a JEM-100B transmission electron microscope at 80 kV accelerating voltage.

Physiological tests were performed in liquid medium MM2 with 0.5 % (v/v) methanol as the sole growth substrate. Growth of isolates was monitored by nephelometry at 600 nm for 2 weeks under a variety of growth conditions, including temperatures of 4–37 °C, pH 2.9–8.0 and NaCl at concentrations of 0.01–5 % (w/v). Variations in pH were achieved by mixing 0.1 M solutions of H_2SO_4 and NaOH. The range of potential growth substrates was examined using 0.05 % (w/v) concentrations of the following carbon sources: formamide, formaldehyde, methylamine, dimethylamine, trimethylamine, glucose, arabinose, xylose, lactose, maltose, rhamnose, raffinose, sucrose, sorbose, fructose, formate, acetate, lactate, malate, oxalate, pyruvate, propionate, succinate, citrate, valerate, capronate, gluconate, glucuronate, mannitol, *myo*-inositol and ethanol. Nitrogen sources were tested by replacing $(NH_4)_2SO_4$ in MM2 with 0.01 % (w/v) KNO_3, $NaNO_2$, urea, glycine, alanine, serine, cysteine, proline, arginine, asparagine or yeast extract.

Cell biomass for cellular fatty acid and isoprenoid quinone analyses and for DNA extraction was obtained from batch cultures grown in liquid medium MM2 with methanol at 24 °C for 10 days. Fatty acid profiles were analysed at the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) as described by Kämpfer & Kroppenstedt (1996). Isoprenoid quinones were extracted according to Collins (1985) and analysed using liquid

chromatography coupled with tandem mass spectrometry (Finnigan LCQ Advantage Max and Mat 8430 system; Thermo Fisher Electronic).

The DNA base composition of strains was determined by thermal denaturation using a Unicam SP1800 spectrophotometer at a heating rate of 0.5 °C min^{-1}. The DNA G+C content was calculated according to Owen *et al.* (1969). PCR-mediated amplification of the 16S rRNA gene was performed using primers 9f and 1492r and reaction conditions described by Weisburg *et al.* (1991). Partial fragments of the *mxaF* gene (encoding the large subunit of methanol dehydrogenase), the *nifH* gene (encoding dinitrogen reductase) and the *rbcL* gene [encoding the large subunit of form I ribulose-1,5-bisphosphate (RuBP) carboxylase/oxygenase (RubisCO)] were amplified using primers and reaction conditions described, respectively, by McDonald & Murrell (1997), Dedysh *et al.* (2004b) and Spiridonova *et al.* (2004). PCR-mediated amplification of the *mmoX* gene was performed using a combination of the previously described forward primer mmoXA (166f) (Auman *et al.*, 2000) and the newly developed reverse primer mmoXmc1 (1353r) (5′-VCGYTCGCCCCARTC-RTC-3′), which was designed to target specifically the *mmoX* sequences of methanotrophs belonging to the *Beijerinckiaceae*, based on all known published sequences plus several unpublished sequences from *Methylocella* isolates in our collection (Dunfield *et al.*, 2010). The PCR protocol consisted of 35 cycles of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and elongation at 72 °C for 1 min, with a final extension step at 72 °C for 7 min. PCR-amplified gene fragments were purified using the Wizard PCR Preps DNA purification system (Promega) and sequenced on an ABI Prism 377 DNA sequencer (PE Applied Biosystems). Phylogenetic analysis was carried out using the ARB program package (Ludwig *et al.*, 2004). Detection of *mmoX* and *mxaF* mRNA transcripts in cells of strain AR4^T after growth on methane, methanol or both substrates together was performed as described for *Methylocystis* sp. H2s by Belova *et al.* (2011) with the modification that RT-PCR of *mmoX* and *mxaF* transcripts was performed using the primer pairs mmoXA/mmoXmc1 (see above) and 1003f/1561r (McDonald & Murrell, 1997).

Small (1–3 mm), opaque, pale-cream, circular colonies with an entire edge and a smooth surface developed on agar medium MM2 after incubation for 1 month at 24 °C. Liquid cultures displayed white turbidity. Flocks of biomass in liquid cultures were formed after 14–16 days of incubation. Cells of strains AR4^T, SOP9 and LAY were Gram-negative, non-motile, straight or slightly curved thin rods, 0.4–0.65 μm wide and 1.1–3.0 μm long. They reproduced by irregular fission and occurred singly (Fig. 1a) or were arranged in rosettes and misshapen cell clusters (Fig. 1b). The formation of rosettes was most pronounced in old (two or more weeks) cultures. No resting cell forms were observed. Overall, this cell morphology was highly similar to that of the recently described facultative methylotroph *Methylovirgula ligni* (Vorob'ev *et al.*, 2009) and was clearly different from cell morphology in *Methylocella* species. Thin sections, however, revealed a cell ultrastructure similar to that observed in members of the genus *Methylocella* (Fig. 1c). The extensive ICM structures typical of pMMO-possessing proteobacterial methanotrophs were absent from cells of strains AR4^T, SOP9 and LAY. Instead, the cells of these isolates contained a vesicular membrane system composed of spherical or ovoid-shaped membrane vesicles located on the periphery of the cytoplasm (Fig. 1c). Intracellular granules of poly-β-hydroxybutyrate were formed at each cell pole.

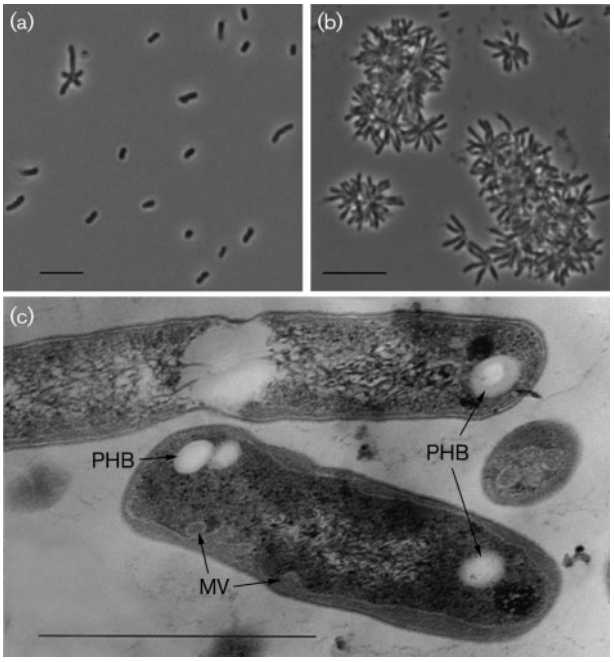

Fig. 1. (a, b) Phase-contrast micrographs of cells of strain AR4^T grown in liquid mineral medium MM2 with methanol for 5 (a) and 14 (b) days. (c) Electron micrograph of an ultrathin section of cells of strain SOP9. MV, Membrane vesicles; PHB, poly-β-hydroxybutyrate. Bars, 5 μm (a, b) and 1 μm (c).

Strains AR4^T, SOP9 and LAY were able to grow on methane as the sole carbon and energy source. However, the preferred growth substrate for these bacteria was methanol (OD_{600}^{max} 0.65; μ 0.015 h^{-1}) rather than methane (OD_{600}^{max} 0.2; μ 0.005 h^{-1}). It was utilized in a wide range of concentrations from 0.01 to 5 %, with an optimum between 0.1 and 1 % (v/v). None of the other C_1 compounds or multicarbon substrates tested supported growth of these strains. The preference for growth on methanol rather than methane is characteristic of pMMO-lacking methanotrophs, i.e. members of the genus *Methylocella*. Indeed, the presence of pMMO in strains AR4^T, SOP9 and LAY could not be confirmed by any of the commonly used approaches. Cells of these isolates did not contain ICM that are present in all pMMO-using methanotrophic proteobacteria. Our attempts to amplify a *pmoA* gene fragment from DNA of

strains AR4^T, SOP9 and LAY using any of the following primer sets for this gene were unsuccessful: A189/A682 (Holmes *et al.*, 1995), A189/Mb661r (Costello & Lidstrom, 1999), A189/Mcap630r and A189/Forest675r (Kolb *et al.*, 2003). In contrast, the colorimetric naphthalene oxidation test (Graham *et al.*, 1992) for sMMO activity in cells of these isolates grown on both copper-free and copper-sufficient media was positive. We were able to amplify *mmoX* gene fragments from DNA of these methanotrophs using the newly designed primer set. Comparative sequence analysis of *mmoX* showed that the novel strains represent a lineage that is distinct from both type I and type II methanotrophs, but falls closer to *Methylocella* (83.3–85.7 % nucleotide sequence identity and 93.2–95.2 % deduced amino acid sequence identity) (Fig. 2). The identity between the deduced MmoX sequences of strains AR4^T, SOP9 and LAY and those from the *Methylosinus*/*Methylocystis* group and type I methanotrophs was 86.6–88.3 and 84.3–86.0 %, respectively.

Comparative sequence analysis of *mxaF*, the gene encoding the large subunit of methanol dehydrogenase, placed the MxaF sequences of our novel isolates in a cluster that was distinct from all previously described MxaF sequences of cultivated methylotrophs (Supplementary Fig. S1, available in IJSEM Online). The most closely related MxaF sequences (86.2–86.8 % identity) were those from the acidophilic methylotroph *Methylovirgula ligni* (Vorob'ev *et al.*, 2009). The identity between the MxaF sequences of strains AR4^T, SOP9 and LAY and MxaF sequences from other known alpha-, beta- and gammaproteobacterial methylotrophs was 67–81, 63–66 and 70–72 %, respectively. It is particularly remarkable that the MxaF sequences of the novel isolates were nearly identical (98.8–100 %) to the inferred peptide sequences of *mxaF* clones designated MeOH1, MeOH2 and M13.3 (GenBank accession numbers AY080938, AY080939 and AF200702) that were retrieved by means of a stable isotope probing technique from different acidic soils amended with ^{13}C-methanol (Radajewski *et al.*, 2000, 2002). Therefore, though these methanotrophs could not be detected by commonly used *mmoX*-targeted approaches, they were identified as metabolically active methylotroph populations in acidic soils by means of an *mxaF*-based approach.

The preference for growth on methanol raised a question concerning sMMO expression in the presence of methanol. To answer this question, we analysed extracts of total RNA from cells of strain AR4^T grown on methane or methanol or in the presence of both substrates for *mmoX* gene expression. *mmoX* mRNA transcripts were detected in cells grown on methane alone and on methane plus methanol, but they were not revealed in methanol-grown bacteria (Supplementary Fig. S2). As expected, *mxaF* mRNA transcripts were detected in all batches of cells examined in this experiment.

Since growth on methanol was more robust, we used extracts of methanol-grown cells to identify the routes of C_1 assimilation in the novel methanotrophs. The novel strains possessed a pyrroloquinoline quinone-containing methanol dehydrogenase that required alkaline pH and ammonium ions for activity *in vitro* with phenazine methosulfate (PMS) as an artificial electron acceptor (Table 1). NAD-dependent formaldehyde and formate dehydrogenases were also detected. High activities of hydroxypyruvate reductase and serine-glyoxylate aminotransferase indicated that the novel isolates assimilate C_1 compounds via the serine pathway. However, low activity of RubisCO was detected as well, suggesting that the RuBP pathway is also employed by these bacteria. Detection and partial sequence analysis of *rbcL*, the gene encoding form I RubisCO, provided genotypic evidence for the presence of

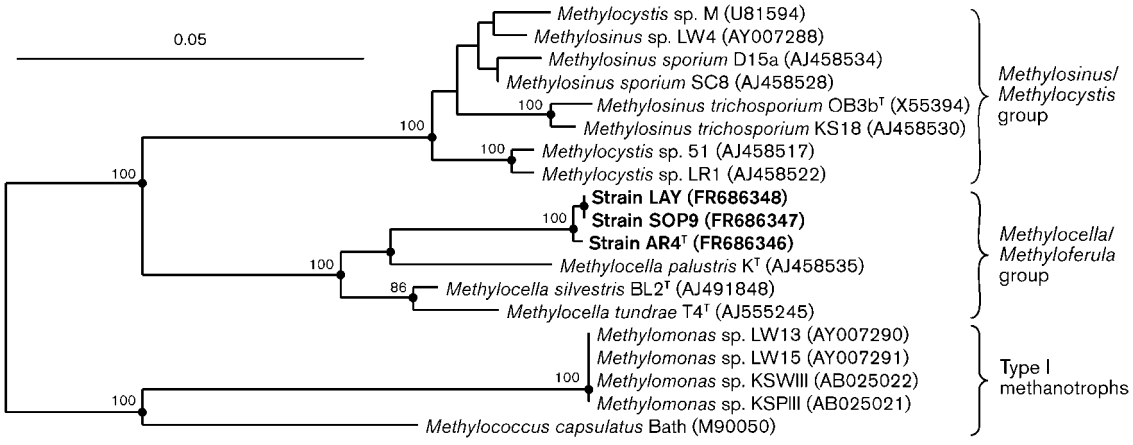

Fig. 2. Unrooted neighbour-joining tree reconstructed on the basis of 368 deduced amino acid sites of partial *mmoX* gene sequences, showing the positions of strains AR4^T, SOP9 and LAY relative to other sMMO-possessing type I and type II methanotrophs. Bootstrap values (1000 data resamplings) >60 % are shown. Filled circles indicate that the corresponding nodes were also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 0.05 substitutions per nucleotide position.

Table 1. Enzyme activities in cell extracts of strain AR4^T grown on methanol

Enzyme	Cofactor(s)	Activity [nmol min^{-1} (mg protein^{-1})]
Methanol dehydrogenase	PMS*	1690
Formaldehyde dehydrogenase	NAD^+	70
Formate dehydrogenase	NAD^+	120
Hydroxypyruvate reductase	NADH, $NADPH_2$	190, 4500
Serine-glyoxylate aminotransferase	NADH, $NADPH_2$	146, 115
Malyl-CoA synthetase/CoA lyase	ATP, CoA	5
RubisCO	–	24

*PMS, Phenazine methosulfate.

an autotrophic metabolism in the novel methanotrophs. The highest nucleotide sequence identity to the *rbcL* gene fragments from these isolates was noted for *Methylocella silvestris* BL2^T (88 % identity) and *Beijerinckia mobilis* (88 %). Of these two organisms, RubisCO activities have been detected only in *Beijerinckia mobilis* (Dedysh *et al.*, 2005b). Since the genome sequence of *Methylocella silvestris* BL2^T confirms the presence of genes encoding RubisCO (Chen *et al.*, 2010), we repeated our analysis of *Methylocella silvestris* BL2^T grown on methanol, but still no RubisCO activity was detected under these growth conditions.

Strains AR4^T, SOP9 and LAY utilized ammonium salts, nitrates, urea and yeast extract as nitrogen sources. They were also capable of slow growth in liquid nitrogen-free medium MM2 under microaerobic conditions (1.0–2.0 % O_2 in the flask headspace). The sequences of *nifH* gene fragments from these methanotrophs displayed highest similarity (97–98 %) to the corresponding gene fragments from acidophilic methanotrophs of the genus *Methylocapsa* and members of the genus *Beijerinckia*.

The isolates were moderately acidophilic and grew in the pH range 3.5–7.2, with optimum growth at pH 4.8–5.2 (Supplementary Fig. S3a). The temperature range for growth was 4–33 °C, with optimum growth at 20–23 °C (Supplementary Fig. S3b). No growth occurred at 37 °C. Growth of strains AR4^T, SOP9 and LAY was inhibited in the presence of NaCl in the medium at concentrations above 0.7 % (w/v).

Table 2. PLFA compositions of strains AR4^T, SOP9 and LAY and phylogenetically related methanotrophs and methylotrophs of the family *Beijerinckiaceae*

Major PLFAs are shown in bold. Values are percentages of total fatty acids. PLFAs that made up <0.1 % of total in all organisms are not included. Data for reference taxa were taken from Dedysh *et al.* (2000, 2004a), Dunfield *et al.* (2003, 2010) and Vorob'ev *et al.* (2009).

Fatty acid	AR4^T	SOP9	LAY	*Methylocella*	*Methylocapsa*	*Methylovirgula ligni*
13 : 0	0	0	0.6	0	0–0.8	0
14 : 0	0.2	0.3	0.2	0–4.1	0	0
iso-15 : 0	0.7	0.9	1.0	0.2–1.2	0.1–0.3	0
16 : 0	4.9	5.1	5.8	3.0–7.7	5.9–7.3	1.8–2.0
10-Methyl 16 : 0	0.4	0.3	0	0	0	0–0.4
16 : 1ω7*c*	2.6	2.6	3.6	**6.8–11.3**	4.7–6.3	0.3–0.4
16 : 1ω7*t*	0	0	0	0–5.8	0	0
16 : 1ω11*c*	0.8	0.8	0.3	0	0	0
iso-17 : 0	0.7	0.9	1.0	0–2.5	0.6–0.9	0–0.2
17 : 0 cyclo	0	0.2	0.2	0–6.5	0	0–0.2
17 : 1ω7*c*	0.3	0.3	0	0	0–1.0	0.2
iso-18 : 0	0	0	0	0–0.5	0	0
18 : 0	0.6	0.6	0.9	0.4–1.2	0.8–7.6	0.8–0.9
18 : 1ω9*c*	0.4	0.3	0	0	0–0.4	0–0.4
18 : 1ω7*c*	**85.7**	**83.2**	**82.3**	**59.2–82.2**	**78.3–81.5**	**87.1–92.8**
19 : 0 cyclo ω8*c*	1.3	3.8	2.5	**0–13.6**	0–2.5	0
19 : 0 cyclo	0	0	0	0	0	1.4–2.6
iso-19 : 0	0	0	0.4	0	0	0
19 : 0	0.9	0.7	0.8	0–0.6	0	0
10-Methyl 19 : 0	0.2	0.2	0.3	0	0	0

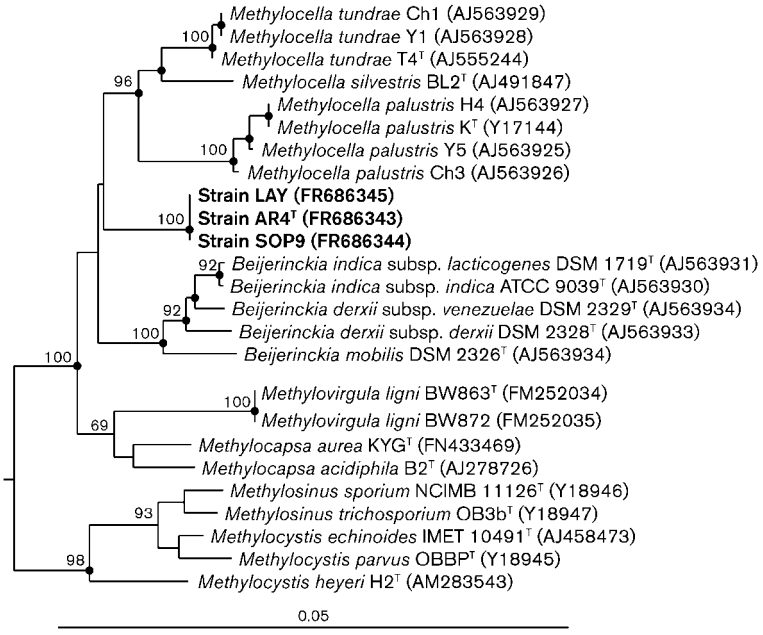

Fig. 3. 16S rRNA gene sequence-based neighbour-joining tree showing the phylogenetic positions of strains AR4^T, SOP9 and LAY in relation to other members of the family *Beijerinckiaceae* and some other methanotrophic representatives of the *Alphaproteobacteria*. Bootstrap values (percentages of 1000 data resamplings) >50 % are shown. Filled circles indicate that the corresponding nodes were also recovered in maximum-likelihood and maximum-parsimony trees. Sequences from the type I methanotrophs *Methylomicrobium album* ACM 3314^T (GenBank accession no. X72777), *Methylobacter luteus* NCIMB 11914^T (AF304195), *Methylomonas methanica* S1^T (AF304196) and *Methylococcus capsulatus* Texas (AJ563935) were used as an outgroup. Bar, 0.05 substitutions per nucleotide position.

Phospholipid fatty acid (PLFA) profiles of the novel methanotrophic isolates were highly similar to those described for methano- and methylotrophic representatives of the family *Beijerinckiaceae*, i.e. the genera *Methylocella*, *Methylocapsa* and *Methylovirgula* (Table 2). The major component of the PLFA profiles of the novel isolates was 11-*cis*-octadecenoic acid (18 : 1ω7*c*), which comprised 83.2–85.7 % of the total fatty acids. Besides 18 : 1ω7*c*, strains AR4^T, SOP9 and LAY contained significant amounts (4.9–5.1 %) of palmitic acid (16 : 0).

A phylogenetic tree constructed on the basis of 16S rRNA gene sequences (Fig. 3) indicated that strains AR4^T, SOP9 and LAY belong to the family *Beijerinckiaceae* of the class *Alphaproteobacteria*. The closest phylogenetic neighbours of these isolates are sMMO-possessing facultative methanotrophs of the genus *Methylocella* (96.4–97.0 % sequence similarity), heterotrophs of the genus *Beijerinckia* (96.5–97.0 %), pMMO-possessing methanotrophs of the genus *Methylocapsa* (96.1–97.0 %) and facultative methylotrophs of the genus *Methylovirgula* (96.1–96.3 %). Members of the *Methylosinus*/*Methylocystis* group displayed only 93.6–94.6 % 16S rRNA gene sequence similarity to strains AR4^T, SOP9 and LAY. The G+C content of the DNA in strains AR4^T, SOP9 and LAY was 55.6–57.5 mol%.

In summary, our novel sMMO-possessing isolates from *Sphagnum* peat bogs and acidic soil possessed a number of characteristics that clearly distinguished them from other currently known methanotrophic members of the family *Beijerinckiaceae* (Table 3). Cell morphology, the absence of ICM and pMMO and the preference for growth on methanol made these strains different from members of the genus *Methylocapsa*. Formation of rosettes, the ability to grow at below pH 4, the inability to utilize multicarbon compounds and the presence of RubisCO activity distinguished strains AR4^T, SOP9 and LAY from *Methylocella* species. Therefore, we propose a novel genus and species, *Methyloferula stellata* gen. nov., sp. nov., to accommodate strains AR4^T, SOP9 and LAY.

Description of *Methyloferula* gen. nov.

Methyloferula [Me.thy′lo.fe′ru.la. N.L. n. *methylum* the methyl group (from French *methyle*, back-formation from French *methylune*, coined from Gr. n. *methu* wine and Gr. n. *hulê* wood); N.L. pref. *methyl-* pertaining to the methyl radical; L. fem. n. *ferula* a rod; N.L. fem. n. *Methyloferula* methyl-using rod].

Gram-negative, aerobic, colourless, non-motile rods that occur singly or are arranged in rosettes and misshapen cell clusters. Reproduce by irregular fission. Colonies are small, circular and smooth. The colony colour varies from white to cream. Produce poly-β-hydroxybutyrate. Mesophilic and moderately acidophilic. Prefer dilute media of low salt content. Obligate utilizers of methane and methanol; the latter is the preferred growth substrate. Possess only a soluble MMO. Capable of atmospheric nitrogen fixation. The major fatty acid is 18 : 1ω7*c*. The major quinone is Q-10. The G+C content of the DNA is 55.6–57.5 mol%. Member of the class *Alphaproteobacteria*, family *Beijerinckiaceae*. The type and only species is *Methyloferula stellata*. Known habitats are acidic peatlands and soils.

Description of *Methyloferula stellata* sp. nov.

Methyloferula stellata (stel.la′ta. L. fem. adj. *stellata* starry).

Description as for the genus plus the following traits. Cells are 0.4–0.65 μm wide and 1.1–3.0 μm long. Carbon

Table 3. Major characteristics that distinguish the novel strains (*Methyloferula* gen. nov.) from other methanotrophs of the family *Beijerinckiaceae*

Data for *Methylocella* were taken from Dedysh *et al.* (2000, 2004a, 2005a) and Dunfield *et al.* (2003) and data for *Methylocapsa* were taken from Dedysh *et al.* (2002) and Dunfield *et al.* (2010).

Characteristic	*Methyloferula* gen. nov.	*Methylocella*	*Methylocapsa*
Cell morphology	Straight or curved rods	Bipolar straight or curved rods	Curved coccoids
Cell size (μm)	0.4–0.65 × 1.1–3.0	0.6–1.0 × 1.0–2.5	0.7–1.2 × 0.8–3.1
Rosette formation	+	−	−
Type of methanotrophy	Obligate	Facultative	Obligate or facultative
Possession of:			
pMMO	−	−	+
sMMO	+	+	−
Preferable growth substrate(s)	Methanol	Methanol, acetate	Methane
Multicarbon compounds utilized	None	Acetate, pyruvate, succinate, malate, ethanol	None or acetate*
RubisCO activity	+	−	−
Growth at/in:			
pH 3.5	+	−	−
0.5 % NaCl	+	−	−
DNA G+C content (mol%)	55.6–57.5	60–63.3	57.3

**Methylocapsa acidiphila* does not utilize multicarbon compounds, while *Methylocapsa aurea* utilizes acetate.

sources include methane and methanol. Grows optimally at methanol concentrations of 0.5–1 %. Nitrogen sources (0.05 %, w/v) include ammonia, nitrate, urea and yeast extract. Capable of growth at pH 3.5–7.2 (optimum pH 4.8–5.2) and at 4–33 °C (optimum 20–23 °C). NaCl inhibits growth at concentrations above 0.7 % (w/v).

The type strain is strain AR4^T (=DSM 22108^T =LMG 25277^T =VKM B-2543^T), which was isolated from the oligo-mesotrophic fen Torfjanoye, Archangelsk region, European North Russia (65° 01′ N 35° 44′ E).

Acknowledgements

This research was supported by the Program 'Molecular and Cell Biology' of the Russian Academy of Sciences, by the RosNauka project no. 02.740.11.0023 and by an Alberta Innovates-Technology Futures New Faculty Award. The authors want to thank N. E. Suzina for electron microscopy and E. N. Detkova for DNA G+C content analysis.

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