Methylocystis rosea sp. nov., a novel methanotrophic bacterium from Arctic wetland soil, Svalbard, Norway (78° N)

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A Gram-negative, rod-shaped, non-motile, non-spore-forming, pink-pigmented bacterium, SV97^T, was isolated from a wetland soil near Ny-Ålesund, Svalbard Islands, Norway (78° N). On the basis of 16S rRNA gene sequence similarity, strain SV97^T was shown to belong to the *Alphaproteobacteria* and was highly related to a number of non-characterized *Methylocystis* strains with GenBank accession nos AJ458507 and AJ458502 (100 %) and AF177299, AJ458510, AJ458467, AJ458471, AJ431384, AJ458475, AJ458484, AJ458501 and AJ458466 (99 %). The most closely related type strains were *Methylocystis parvus* OBBP^T (97.2 %) and *Methylocystis echinoides* IMET 10491^T (97 %). The closest related recognized species within the genus *Methylosinus* was *Methylosinus sporium* NCIMB 11126^T (96.0 % similarity). Chemotaxonomic and phenotypic data (C_{18:1}ω8 as the major fatty acid, non-motile, no rosette formation) supported the affiliation of strain SV97^T to the genus *Methylocystis*. The results of DNA–DNA hybridization and physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain SV97^T from the two recognized *Methylocystis* species. Strain SV97^T therefore represents a novel species, for which the name *Methylocystis rosea* sp. nov. is proposed, with the type strain SV97^T (=DSM 17261^T = ATCC BAA-1196^T).

The type II methanotrophic bacteria consist of four genera, *Methylocystis* and *Methylosinus* (Bowman *et al.*, 1993), *Methylocella* (Dedysh *et al.*, 2000) and *Methylocapsa* (Dedysh *et al.*, 2002). They are distinguished from type I methanotrophic bacteria by genetic and phenotypic features. Type II methanotrophs belong to the *Alphaproteobacteria*, assimilate formaldehyde by the serine pathway, have intracytoplasmic membranes aligned parallel to the cell wall (*Methylocystis* and *Methylosinus*), membrane vesicles packed in parallel on one side of the cell membrane (*Methylocapsa*) or a vesicular membrane system connected to the cytoplasmic membrane (*Methylocella*), and the major fatty acid is C_{18:1}ω8c

(*Methylocystis* and *Methylosinus*) (Hanson & Hanson, 1996) or C_{18:1}ω7c (*Methylocella* and *Methylocapsa*) (Dedysh *et al.*, 2000, 2002). At present, the genus *Methylocystis* comprises two species with validly published names, *Methylocystis parvus* (Bowman *et al.*, 1993; Whittenbury *et al.*, 1970) and *Methylocystis echinoides* (Galchenko *et al.*, 1977). However, a large number of strains designated as members of the genus *Methylocystis* has been isolated from several different environments (Dunfield *et al.*, 2002; Heyer *et al.*, 2002; Wise *et al.*, 1999).

Strain SV97^T was isolated from a soil core collected from a high Arctic wetland near the Ny-Ålesund settlement (78° 56' N 11° 53' E) on the Svalbard Islands, Norway, in July 1997. At the time of sampling, the soil had a pH of 6.4 and the temperature of the soil was 10 °C at the surface and 5 °C at 10 cm below the surface and the permafrost level was at 25 cm. Methane emission from the same site was determined in July and September 1998 and in July and August 1999 and emission varied from 93 to 2801 µg CH₄ m⁻² h⁻¹ (Høj *et al.*, 2005).

After removal of the fresh vegetation layer, the upper 10 cm of the soil core was mixed and 2 g soil was added to 10 ml nitrate mineral salt (NMS) medium (Whittenbury *et al.*,

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Abbreviation: PHB, poly-β-hydroxybutyrate.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *pmoA* and *nifH* gene sequences of *Methylocystis rosea* SV97^T are AJ414656, AJ414657 and DQ010107, respectively.

A chart showing the growth characteristics of *Methylocystis rosea* SV97^T at different temperatures and a detailed description of the methods used for fatty acid analysis are available as supplementary material in IJSEM Online.

1970) at pH 6.8 and shaken for 10 min at 200 r.p.m. After 10 min of sedimentation, 1 ml supernatant was mixed with 9 ml NMS medium in a 120 ml serum bottle (Alltech) and then sealed with a rubber stopper and crimp cap. The gas atmosphere inside the bottle was enriched with methane by replacing 20 ml air with 20 ml CH₄/CO₂ (95:5) mix. The bottle was incubated at 20 °C and subcultured every 2–3 weeks (1 ml culture to 9 ml fresh NMS medium). After four or five subculturing steps in liquid media, bacteria were plated on NMS medium containing 1.5 % noble agar (bacteriological agar type E; Biokar Diagnostics). The plates were incubated at 20 °C in sealed chambers containing approximately 35 % methane in air. Colonies were picked and restreaked. Heterotrophic contamination was checked by streaking colonies on agar plates with rich medium, containing 0.5 % tryptone, 0.25 % yeast extract, 0.1 % glucose and 2.0 % agar. These plates were incubated at 20 °C without additional methane. The cultures were considered to be pure when only one cell type was observed under light microscopy and no growth on nutrient-rich medium or on NMS agar without methane was observed. Exospore formation was assayed by determining cell viability after heating 3-week-old cultures to 80 °C for 20 min and then observing microcolony formation on NMS agar after 2 weeks incubation with methane (Bowman *et al.*, 1993). Cyst formation

was assayed by the method of Vela & Wyss (1964) and by light microscopy of 3-week-old cultures. The presence of poly-β-hydroxybutyrate (PHB) inclusions was determined by staining with 0.03 % (w/v) Sudan black and 0.5 % safranin (Gerhardt, 1981) and observing using phase-contrast microscopy. The presence of PHB was also confirmed by chemical analysis (Gerhardt, 1981). Tolerance of NaCl concentrations ranging from 0.01 to 1.0 % (w/v) was determined with NMS agar cultures. Growth at pH range 5.5–9.0 was determined in liquid cultures of NMS. The pH of the medium was adjusted with NaOH or HCl. Growth on alternative carbon sources was determined on methanol concentrations ranging from 0.1 to 1.0 % (v/v) and ethanol, formate, formamide, formaldehyde, glucose, sucrose, acetate, citrate and yeast extract, all at 0.1 % (v/v), in liquid cultures of NMS. Ability to fix nitrogen was determined by growth on nitrate (NMS), ammonia (AMS) and nitrogen-free (NFMS) media (Auman *et al.*, 2001). Possession of the soluble methane monooxygenase (sMMO) was tested for by using a colorimetric assay described by Brusseau *et al.* (1990), with modifications (Graham *et al.*, 1992). The DNA G + C content was determined by thermal denaturation using a spectrophotometer (Cary 4E; Varian) at a heating rate of 0.5 °C min⁻¹. The DNA G + C content was calculated as described by Mandel *et al.* (1970). DNA–DNA hybridization

Fig. 1. Phylogenetic relationship of the 16S rRNA gene sequences of *Methylocystis rosea* SV97^T and other *Methylocystis* strains and related genera. The dendrogram shows the results of an analysis in which DNADIST and neighbour-joining were used. Bootstrap values greater than 50 %, derived from 100 replicates, are also shown. Bar, 1 % sequence divergence.

was performed with *Methylocystis parvus* OBBP^T and *Methylocystis echinoides* IMET 10491^T according to Ezaki *et al.* (1989). The 16S rRNA gene, soluble methane monooxygenase gene (*mmoX*), small subunit of particulate methane monooxygenase gene (*pmoA*) and nitrogenase reductase structural gene (*nifH*) were analysed by PCR as described previously (Fuse *et al.*, 1998; Míguez *et al.*, 1997).

Growth of strain SV97^T was measured at temperatures ranging from 5 to 40 °C, using a temperature gradient apparatus. Cultures of SV97^T were prepared by adding 5 ml starting culture to 27 ml serum bottles. The bottles were sealed with rubber stoppers and crimp caps before 5 ml air was replaced with 5 ml CH₄/CO₂ (95:5) mix and the cultures were grown for 5 days. The OD₆₀₀ was measured for the starting culture and for the 5-day-old cultures using a Spectramax 250 microplate spectrophotometer system (Molecular Devices). The maximum OD₆₀₀ of strain SV97^T was 0.7 and was measured after 15 days at 20 °C. The experiment was repeated with three replicates at each temperature setting and net growth was calculated by subtracting the OD₆₀₀ of the starting culture from the OD₆₀₀ values of the 5-day-old cultures. The mean values for net growth and standard deviations were calculated.

Phylogenetic analysis of the 16S rRNA gene sequence was performed using the PHYLIP (Felsenstein, 1993) and TREEVIEW (Page, 1996) software packages after multiple alignment of data by CLUSTAL_X (Thompson *et al.*, 1997). Distances (Kimura two-parameter model) and clustering by neighbour-joining methods were determined by using bootstrap values based on 100 replicates (Fig. 1). Phylogenetic analysis and alignment of *pmoA* data were performed using the software package ARB (Ludwig *et al.*, 2004) (Fig. 2).

The 16S rRNA gene sequence of strain SV97^T was a continuous stretch of 1438 bp. Sequence similarity calculations indicated that the closest relatives of strain SV97^T with validly published names were *Methylocystis parvus* OBBP^T (97.2 %) and *Methylocystis echinoides* IMET 10491^T (97 %). High sequence similarities were found with many non-characterized strains of *Methylocystis* with GenBank accession nos AJ458507 and AJ458502 (100 %) and AF177299, AJ458510, AJ458467, AJ458471, AJ431384, AJ458475, AJ458484, AJ458501 and AJ458466 (99 %) (Table 1). Lower sequence similarity was found with members of the genus *Methylosinus*. *Methylosinus sporium* NCIMB 11126^T and *Methylosinus trichosporium* OB3b^T both showed 96 % sequence similarity to SV97^T. Sequence similarity calculations

Fig. 2. Phylogenetic relationship of the *pmoA* sequences of *Methylocystis rosea* SV97^T and other *Methylocystis* strains and related genera. The dendrogram shows the results of an analysis in which PROTDIST and neighbour-joining were used. Bootstrap values greater than 50 %, derived from 100 replicates, are also shown. Bar, 10 % sequence divergence.

indicated that the closest related *nifH* sequences were derived from *Methylocystis echinoides* IMET 10491^T (97 %), '*Methylocystis minimus*' INMI 41 (97 %) and *Methylocella silvestris* BL2 (91 %).

Cells of strain SV97^T were non-motile, 0.8–1.1 µm wide and 1.1–2.5 µm in length. Cells often appeared singly or in aggregates, but never in rosettes. Colonies were pink–red pigmented (Fig. 3). Strain SV97^T grew at all pH values tested, from 5.5 to 9.0, and no exospores or cysts were revealed using the methods described. Strain SV97^T was obligately methane oxidizing; it did not grow on any of the alternative carbon sources tested. Strain SV97^T grew well on both NMS and AMS media and grew poorly on NFMS. The presence of *nifH* (Poly *et al.*, 2001) indicates that strain SV97^T actively fixes nitrogen (Auman *et al.*, 2001). A type II intracytoplasmic membrane structure, with pairwise membranes aligned parallel to the cell wall, was confirmed using transmission electron microscopy (Fig. 4). Staining with

Sudan black, phase-contrast microscopy and chemical analysis indicated the presence of PHB inclusions. The major phospholipid fatty acids for strain SV97^T were C_{18:1}ω8 (54.2 %), C_{18:1}ω7 (39.7 %) and C_{16:1}ω7 (6.1 %). Details of the methods used for fatty acid analysis are available as Supplementary material in IJSEM Online. Isolate SV97^T had a DNA G + C content of 62 mol% (± 1 %). Strain SV97^T tested negative for *mmoX* in PCR assays and no sMMO was detected by the colorimetric assay. Isolate SV97^T had an optimum growth temperature of 27 °C, but grew well at temperatures from 14 to 37 °C (see Supplementary Fig. S1 in IJSEM Online). DNA–DNA hybridization between strain SV97^T and *Methylocystis parvus* OBBP^T and *Methylocystis echinoides* IMET 10491^T revealed 11 and 12 % DNA–DNA relatedness, respectively. Strain SV97^T differs from *M. parvus* OBBP^T in several phenotypic and genotypic characteristics; it does not have coccibacillus-shaped cells but has rod-shaped cells, does not produce cysts, has pink–red-pigmented cells, has no urease activity, has a lower DNA

Table 1. 16S rRNA gene sequence similarities of *Methylocystis* isolates compared with strain SV97^T

Unusual phenotypes of strains and the presence of *mmoX* are included. Strain SV97^T shows 97 % gene sequence similarity to *M. echinoides* IMET 10491^T and *M. parvus* OBBP^T. The GenBank accession numbers of the strains are shown in Fig. 1. +, Positive; –, negative; ND, not determined.

Strain	Number of nucleotides	Similarity (%)	<i>mmoX</i>	Unusual phenotypes	Reference
<i>M. rosea</i> SV97 ^T	1438	–	–	Dark pink	This study
<i>M. echinoides</i> strain 2	1393	100	–		Heyer <i>et al.</i> (2002)
' <i>Methylocystis minimus</i> ' 82	1407	100	+		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. AML-A6	1431	99	–	White, coccus	Wise <i>et al.</i> (1999)
<i>Methylocystis</i> sp. 50/54	1402	99	+		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. 495/4	1400	99	+		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. CSC-1	1422	99	ND		D. Hudson, I. Uz, A. V. Ogram and A. S. Lindner (unpublished)
<i>Methylocystis</i> sp. IMET 10486	1406	99	+		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. SC2	1409	99	–	White	Dunfield <i>et al.</i> (2002)
<i>Methylocystis</i> sp. 51	1402	99	+		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. 50/42a	1381	99	+		Heyer <i>et al.</i> (2002)
' <i>Methylocystis methanolicus</i> ' 39	1403	99	–		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. 62/12	1407	99	–		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. WRS	1443	98	ND		M. S. Vecherskaya, P. van Bodegom, E. G. Zoetendal and A. J. M. Stams (unpublished)
<i>Methylocystis</i> sp. AML-A3	1462	98	–	Pink, coccus	Wise <i>et al.</i> (1999)
<i>Methylocystis</i> sp. KS33	1405	98	–	Coccoid	Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. 42/22	1408	98	–		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. IMET 10489	1404	98	–		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. M31	1407	98	–		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. M42/1	1401	98	–		Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. KS7	1407	98	–	Large coccus and rods, motile	Heyer <i>et al.</i> (2002)
<i>Methylocystis</i> sp. KS8a	1406	98	–	Coccoid and bent rods, motile	Heyer <i>et al.</i> (2002)

Fig. 3. Differences in pigmentation between *M. echinoides* IMET 10491^T (left), *M. parvus* OBBP^T (middle) and *M. rosea* SV97^T (right), grown on the same NMS agar plate.

G+C content (62 mol%) and shows 11 % DNA–DNA hybridization and 97.2 % 16S rRNA gene sequence similarity. Strain SV97^T differs from *Methylocystis echinoides* IMET 10491^T in that it does not have coccibacillus-shaped cells, does not show spinae, has pink–red-pigmented cells, grows at pH 5.5 and 9.0, produces PHB and shows only 12 % DNA–DNA hybridization and 97 % 16S rRNA gene sequence similarity. These differences are summarized in Table 2.

Fig. 4. Transmission electron micrograph of a cell of strain SV97^T showing the type II intracytoplasmic membranes aligned with the periphery of the cell and inclusions (white spot). Bar, 200 nm.

Strain SV97^T groups very closely with a number of *Methylocystis* isolates which have not been fully characterized (*Methylocystis* spp. 51, 50/54, 495/4, ‘*Methylocystis minimus*’ 82 and *Methylocystis echinoides* 2) (Fig. 1) and which were isolated from a variety of different environments, including Baltic coast sediment, soil near an oil extraction plant and bog water (Heyer *et al.*, 2002). From the data available (Table 1), it can be seen that none of these strains, with the exception of SV97^T, are pink pigmented. One strain, *Methylocystis echinoides* 2 (Galchenko *et al.*, 1977), has spinae, the other four strains have no particular distinguishing phenotype, but are all *mmoX*-positive. These strains may well be representatives of the same species as SV97^T, but this would need to be confirmed by further characterization and DNA–DNA hybridization studies. In view of the phenotypic and genotypic differences between strain SV97^T and the other *Methylocystis* type species (including 16S rRNA gene sequences, DNA–DNA hybridization and phenotypic characteristics), we propose that strain SV97^T, isolated from wetland soil from the Svalbard Islands, Norway, represents a novel species with the name *Methylocystis rosea* sp. nov.

According to Rule 65 (2) of the Bacteriological Code (Lapage *et al.*, 1992), the genus *Methylocystis* is incorrectly in

Table 2. Characteristics that distinguish *Methylocystis rosea* SV97^T from other type strains of the genus *Methylocystis*

Species: 1, *M. rosea* SV97^T; 2, *M. echinoides* IMET 10491^T; 3, *M. parvus* OBBP^T. Data for *M. echinoides* IMET 10491^T and *M. parvus* OBBP^T are from Bowman *et al.* (1993). All type strains of the genus *Methylocystis* have reniform or vibrioid cell morphology, type II membranes, are non-motile, grow at 28 °C, assimilate carbon via the serine pathway, are oxidase-positive, contain the *nifH* gene and have C18:1ω8 as the major phospholipid fatty acid.

Characteristic	1	2	3
Cell morphology:			
Coccibacilli	–	+	+
Rods	+	+	–
Spinae	–	+	–
Cyst formation	–	–	+
Pigmentation:			
White or buff	–	+	–
Diffusible brown	–	–	+
Pale pink	–	–	+
Pink–red	+	–	–
Growth at:			
37 °C	+	–	+
pH 9.0	+	–	+
pH 5.0	+	–	+
Biochemistry:			
PHB	+	–	+
Urease	–	–	+
DNA G+C content (mol%)	62	62	64–67
DNA–DNA hybridization with SV97 ^T (100 %)		12 %	11 %

the masculine gender. As the Greek noun *kustis* is in the feminine gender, the Neo-Latin name *Methylocystis* should also be in the feminine gender. In turn, the specific epithet of the type species, *Methylocystis parvus*, should correctly be *parva*. The specific epithet *echinoides* [*Methylocystis echinoides* (Bowman *et al.*, 1993; Galchenko *et al.*, 1977)] is correct, but is a Neo-Latin feminine adjective.

Description of *Methylocystis rosea* sp. nov.

Methylocystis rosea (ro'se.a. L. fem. adj. *rosea* rose-coloured, rosy).

Cells are Gram-negative, polymorphic, straight or curved fat rods, 1·1–2·5 µm long and 0·8–1·1 µm wide. Colonies on NMS agar plates are pink–red pigmented. Cells occur singly or in shapeless aggregates, but do not form rosettes when observed by light microscopy. The cells are non-motile and possess a type II intracellular membrane system. Cells do not possess sMMO, but have *nifH*. Cells are catalase-positive. Grows at temperatures from 5 to 37 °C. Optimal growth occurs at 27 °C, no growth occurs at 40 °C. Does not require NaCl for growth and cells are not lysed by 2% SDS. Grows well at pH from 5·5 to 9·0. Phospholipid fatty acids are C_{18:1ω8} (54·2%), C_{18:1ω7} (39·7%) and C_{16:1ω7} (6·1%). No exospores or cysts have been revealed. Accumulates PHB. The DNA G+C content is 62 mol%. The type strain shows DNA–DNA hybridization values of 11 and 12%, respectively, to *Methylocystis parvus* OBBP^T and *Methylocystis echinoides* IMET 10491^T.

The type strain, SV97^T (= ATCC BAA-1196^T = DSM 17261^T), was isolated from a high Arctic wetland soil near Ny-Ålesund, Svalbard Islands, Norway.

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References

- Auman, A. J., Speake, C. C. & Lidstrom, M. E. (2001). *nifH* sequences and nitrogen fixation in type I and type II methanotrophs. *Appl Environ Microbiol* **67**, 4009–4016.
- Bowman, J. P., Sly, L. I., Nichols, P. D. & Hayward, A. C. (1993). Revised taxonomy of the methanotrophs: description of *Methylobacter* gen. nov., emendation of *Methylococcus*, validation of *Methylosinus* and *Methylocystis* species, and a proposal that the family *Methylococcaceae* includes only the group I methanotrophs. *Int J Syst Bacteriol* **43**, 735–753.
- Brusseau, G. A., Tsien, H. C., Hanson, R. S. & Wackett, L. P. (1990). Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. *Biodegradation* **1**, 19–29.
- Dedysh, S. N., Liesack, W., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Bares, A. M., Panikov, N. S. & Tiedje, J. M. (2000). *Methylocella palustris* gen. nov., sp. nov., a new methane-oxidizing acidophilic bacterium from peat bogs, representing a novel subtype of serine-pathway methanotrophs. *Int J Syst Evol Microbiol* **50**, 955–969.
- Dedysh, S. N., Khmelenina, V. N., Suzina, N. E., Trotsenko, Y. A., Semrau, J. D., Liesack, W. & Tiedje, J. M. (2002). *Methylocapsa acidiphila* gen. nov., sp. nov., a novel methane-oxidizing and dinitrogen-fixing acidophilic bacterium from *Sphagnum* bog. *Int J Syst Evol Microbiol* **52**, 251–261.
- Dunfield, P. F., Yimga, M. T., Dedysh, S. N., Berger, U., Liesack, W. & Heyer, J. (2002). Isolation of a *Methylocystis* strain containing a novel *pmoA*-like gene. *FEMS Microbiol Ecol* **41**, 17–26.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorimetric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1993). PHYLIP – phylogenetic interference package, version 3.5c. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, USA.
- Fuse, H., Ohta, M., Takimura, O., Murakami, K., Inoue, H., Yamaoka, Y., Oclarit, J. M. & Omori, T. (1998). Oxidation of trichloroethylene and dimethyl sulfide by a marine *Methylobacterium* strain containing soluble methane monooxygenase. *Biosci Biotechnol Biochem* **62**, 1925–1931.
- Galchenko, V. F., Shiskina, V. N., Suzina, N. E. & Trotsenko, Y. A. (1977). Isolation and properties of new strains of obligate methanotrophs. *Mikrobiologiya* **46**, 723–728.
- Gerhardt, P. (1981). *Manual of Methods for General Bacteriology*. Washington, DC: American Society for Microbiology.
- Graham, D. W., Korich, D. G., LeBlanc, R. P., Sinclair, N. A. & Arnold, R. G. (1992). Applications of a colorimetric plate assay for soluble methane monooxygenase activity. *Appl Environ Microbiol* **58**, 2231–2236.
- Hanson, R. S. & Hanson, T. E. (1996). Methanotrophic bacteria. *Microbiol Rev* **60**, 439–471.
- Heyer, J., Galchenko, V. F. & Dunfield, P. F. (2002). Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. *Microbiology* **148**, 2831–2846.
- Høj, L., Olsen, R. A. & Torsvik, V. L. (2005). Archaeal communities in high Arctic wetlands at Spitsbergen, Norway (78° N) as characterized by 16S rRNA gene fingerprinting. *FEMS Microbiol Ecol* **53**, 89–101.
- Lapage, S. P., Sneath, P. H. A., Lessel, E. F., Skerman, V. B. D., Seeliger, H. P. R. & Clark, W. A. (editors) (1992). *International Code of Nomenclature of Bacteria (1990 Revision). Bacteriological Code*. Washington, DC: American Society for Microbiology.
- Ludwig, W., Strunk, O., Westram, R. & 29 other authors (2004). ARB: a software environment for sequence data. *Nucleic Acids Res* **32**, 1363–1371.
- Mandel, M., Igambi, L., Bergendahl, J., Dodson, M. L., Jr & Scheltgen, E. (1970). Correlation of melting temperature and cesium chloride buoyant density of bacterial deoxyribonucleic acid. *J Bacteriol* **101**, 333–338.
- Miguez, C. B., Bourque, D., Sealy, J. A., Greer, C. W. & Groleau, D. (1997). Detection and isolation of methanotrophic bacteria possessing soluble methane monooxygenase (sMMO) genes using the polymerase chain reaction (PCR). *Microb Ecol* **33**, 21–31.

- Page, R. D. M. (1996).** TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12**, 357–358.
- Poly, F., Monrozier, L. J. & Bally, R. (2001).** Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* **152**, 95–103.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997).** The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Vela, G. R. & Wyss, O. (1964).** Improved stain for visualization of *Azotobacter* encystment. *J Bacteriol* **87**, 476–477.
- Whittenbury, R., Phillips, K. C. & Wilkinson, J. F. (1970).** Enrichment, isolation and some properties of methane-utilizing bacteria. *J Gen Microbiol* **61**, 205–218.
- Wise, M. G., McArthur, J. V. & Shimkets, L. J. (1999).** Methanotroph diversity in landfill soil: isolation of novel type I and type II methanotrophs whose presence was suggested by culture-independent 16S ribosomal DNA analysis. *Appl Environ Microbiol* **65**, 4887–4897.