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REVIEW

Exploring Methanotroph Diversity in Acidic Northern Wetlands: Molecular and Cultivation-Based Studies[1]

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Abstract—Acidic wetlands of the northern hemisphere are an important source of methane, a major greenhouse gas. The taxonomic identity of the aerobic methanotrophic bacteria, which colonize these environments and reduce the potential flux of methane to the atmosphere, has remained elusive for a long time. Both cultivation-independent molecular approaches and cultivation-based studies have been used to identify methanotrophs in this acidic habitat. It was shown that acidic peat is colonized mainly by methanotrophic representatives of the *Alphaproteobacteria*: *Methylocystis* spp., *Methylocella* spp. and *Methylocapsa* spp. Novel methanotrophic isolates from acidic wetlands display a number of unique characteristics and metabolic traits including unusual cell ultrastructure and fatty acid composition, ability to utilize some multicarbon compounds as growth substrates, and new regulatory mechanisms of methane oxidation. Several other methanotroph populations, which have been detected in acidic peat by molecular approaches but have so far eluded isolation, represent a challenge for further cultivation studies.

Key words: methanotrophic bacteria, acidic wetlands, molecular ecology techniques, cultivation, *Methylocella*, *Methylocystis, Methylocapsa*.

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1. INTRODUCTION

Northern wetlands are an important source of the greenhouse gas methane [1–5]. Acidic peat bogs are the most extensive type of wetland, occupying about 3% of total land area and being one of the dominant terrestrial ecosystems in the boreal forest zone of North America and Eurasia. A substantial part of the organic matter decomposition in wetlands occurs under anaerobic conditions with the consequent production of methane. The major barrier that limits the release of CH_4 from peatlands is its in situ consumption by indigenous aerobic methanotrophic bacteria in the oxic peat layers [6]. These bacteria inhabit a spectrum of diverse environments and have the unique ability to use CH_4 as a sole carbon and energy source [7]. High activity of methane oxidation in the oxic layers of acidic northern wetlands was recognized many years ago [8–14]. Recently, the evidence for anaerobic CH_4 oxidation in freshwater northern peatlands was also reported, suggesting that this process may be an important control on methane fluxes in these ecosystems [15]. Experiments using a variety of potential electron acceptors demonstrated that Fe(III) and SO_4^{2-} are not quantitatively important, while the role of NO_3^- or some organic molecules derived from organic matter decay is uncertain and deserves more attention. The exact mechanisms for anaerobic methane oxidation and the microorganisms responsible for this process in peatlands remain unclear and, therefore, are not discussed in this review.

2. CURRENTLY DESCRIBED DIVERSITY OF AEROBIC METHANOTROPHS

At present, methanotrophic capabilities are recognized in members of two bacterial phyla, the *Proteobacteria* and *Verrucomicrobia* [7, 16]. The latter were described in 2007–2008 and their diversity and distribution in nature have not yet been analyzed. In contrast, the biology and ecology of methanotrophs in the *Proteobacteria* have been studied extensively over the past 40 years [7, 17, 18]. These methanotrophs are divided into two groups, type I and type II, which belong to the *Gamma-* and *Alphaproteobacteria*, respectively. Members of these groups differ with regard to their cellular ultrastructure, C1-utilization pathway, fatty acid composition, and other physiological and biochemical characteristics. The list of taxonomically described methanotrophs includes now 14 genera, which belong to the families *Methylococcaceae, Methylocystaceae* and *Beijerinckiaceae* (Fig. 1). The ability to oxidize methane was recently identified in two filamentous, sheathed gammaproteobacteria, *Crenothrix polyspora*

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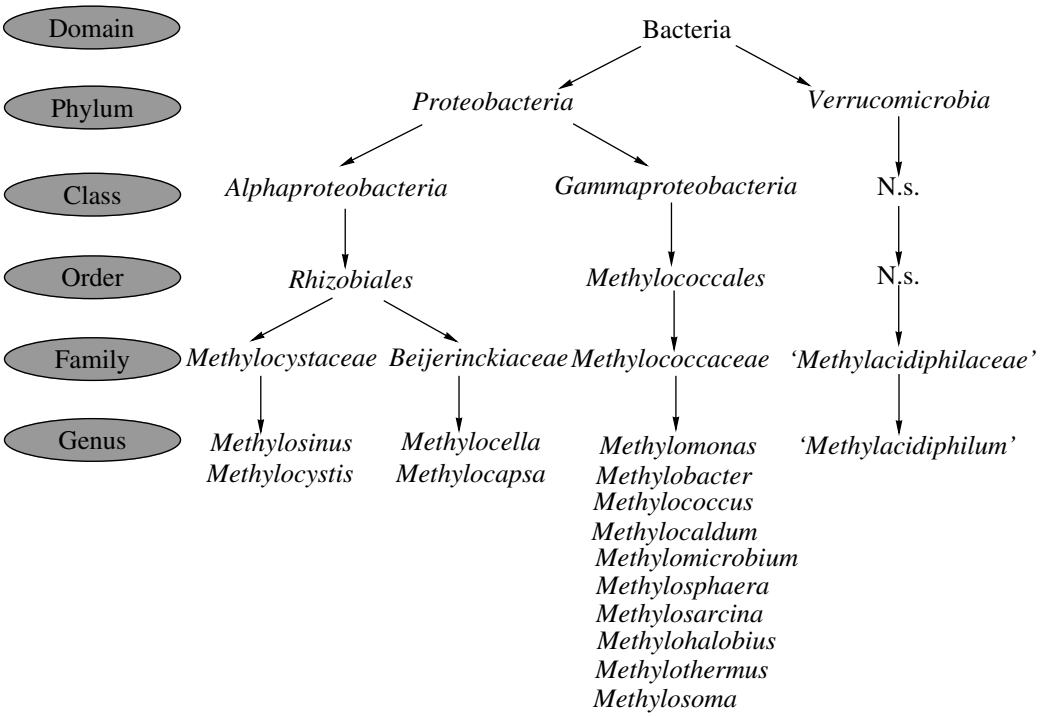

Fig. 1. Currently described diversity of aerobic methanotrophic bacteria. N.s., not specified.

and *Clonothrix fusca* [19, 20], but these unique organisms have not yet been isolated into pure culture.

The use of enzymes known as methane monooxygenases to catalyze the oxidation of methane to methanol is a defining characteristic of methanotrophs [7]. There are two forms of this enzyme: a particulate membrane form (pMMO) and a soluble cytoplasmic form (sMMO). The pMMO has been found in all known methanotrophs except for the genus *Methylocella* [21, 22], while the sMMO is present in *Methylocella* and a several type I and type II methanotroph species [23]. The biochemistry of these two enzymes has been studied in detail (reviewed in [24, 25]).

All MMO-possessing methanotrophs are obligately aerobic bacteria, which inhabit a wide range of natural environments of diverse temperature, salinity and pH. In contrast, most methanotrophs available from the culture collections are mesophilic and neutrophilic, although acidophilic, alkaliphilic, psychrophilic and thermophilic species have also been described (reviewed in [16, 26, 27]).

Northern *Sphagnum*-dominated wetlands are acidic (pH 3.5–5.5) and cold environments. Other characteristic features of this habitat are the low content of mineral elements in peat water (5 to 50 mg per liter), the absence of available nitrogen, and the presence of inhibitory products from mosses. The identity of the methanotrophic bacteria that colonize these hostile environments remained unknown for a long time. Last decade, however, significant breakthroughs were made in molecular identification and isolation of peat-inhabiting methanotrophs. The milestones in this research are shown in Fig. 2 and are reviewed below.

3. APPLICATION OF MOLECULAR TECHNIQUES FOR THE IDENTIFICATION AND QUANTIFICATION OF PEAT-INHABITING METHANOTROPHS

The ecological application of molecular techniques has opened up a new opportunity for direct detection of methanotrophic bacteria in environmental samples [28, 43]. Both phylogenetic (16S rRNA gene) and functional genes of methanotrophs can be used as molecular markers to study ecology of these bacteria. Functional marker genes have a clear advantage of being exclusively specific for the particular functional group of organisms and allowing detection of putative uncultivated members of this group based on the presence of a homologous gene sequence. In the case of methanotrophs, two genes, *pmoA* and *mmoX*, encoding the α-subunit of pMMO and the α-subunit of the sMMO hydroxylase, respectively, are of particular use in molecular ecology studies. Other functional gene markers, which are not unique to the methanotrophs but can be used to identify these bacteria, are *mxaF* (encoding the large subunit of methanol dehydrogenase) and *nifH* (encoding dinitrogenase reductase). Currently, a wide variety of primers and probes targeting 16S rRNA genes of different methanotroph groups as well as *pmoA*, *mmoX* and *mxaF* genes is available [43].

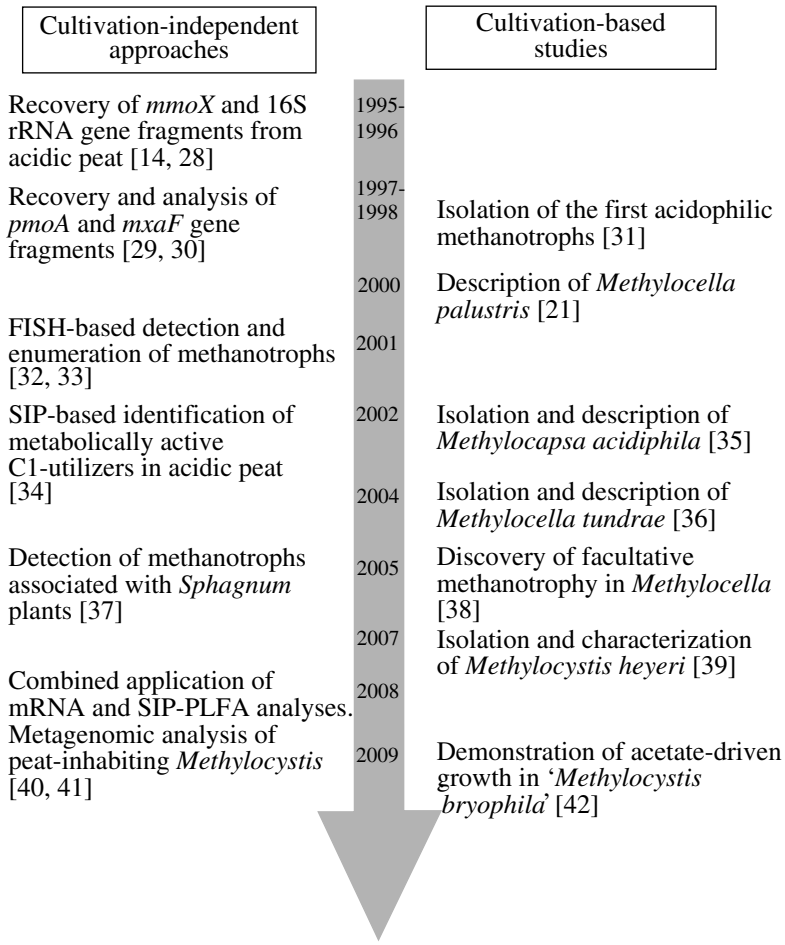

Fig. 2. Milestones in studying methanotroph diversity in acidic northern wetlands.

The very first attempt to use *mmoX*-targeted primers has shown the predicted PCR products from DNA from acidic peat, suggesting that this habitat is abundantly colonized by methanotrophic bacteria [28]. Other evidence for the existence of acidophilic methanotrophs was obtained by screening 16S rRNA gene clone libraries from several peat samples by means of hybridization with specific probes [14]. A few of these clones were found to be representatives of a potentially novel group of methanotrophs related to the *Methylosinus*/*Methylocystis* cluster. Further PCR-mediated retrieval of *pmoA* and *mxaF* gene fragments from a blanket peat bog showed that the corresponding sequences are quite distinct for this environment and are somewhat related to those of type II methanotrophs but form a distinct phylogenetic cluster [29, 30]. It has been suggested that these sequences may originate from a novel group of acidophilic methanotrophs which have yet to be cultured. As we know now, these *pmoA* and *mxaF* gene sequences are representative for peat-inhabiting *Methylocystis* spp., which are the most abundant methanotroph group in this acidic environment.

Further research into methanotroph diversity in acidic northern wetlands was composed of both molecular and cultivation-based studies, which greatly complemented each other (Fig. 2). As soon as the isolates of acidophilic methanotrophs *Methylocella* and *Methylocapsa* became available, new primers and probes were designed for the specific detection of these bacteria. Vice versa, a recovery of any novel methanotroph-affiliated nucleotide sequences from acidic peat always stimulated new isolation attempts.

Several molecular techniques that have been successfully applied for the identification and quantification of peat-inhabiting methanotrophs are discussed below.

Fluorescence in situ hybridization. Fluorescence in situ hybridization (FISH) allows the specific detection and enumeration of target populations directly in their natural environment [44, 45]. Oligonucleotide probes that have been developed for FISH-mediated detection of methanotrophic bacteria are listed in table. FISH was successfully applied to determine the abundance of distinct phylogenetic groups of methanotrophs in acidic (pH 4.2) *Sphagnum* peat from West Siberia

and northern Germany [32, 33]. The total numbers of methanotrophs detected by FISH were $3.1 \pm 0.2 \times 10^6$ and $5.7 \pm 0.4 \times 10^6$ cells g^{-1} (wet weight) of peat in *Sphagnum* peat samples from West Siberia and northern Germany, respectively. The numerically largest methanotroph group, accounting for 60 and 96% of total methanotroph cells detected in both wetland sites, was *Methylocystis* spp., belonging to a phylogenetic sub-group of *Methylocystis* detectable by probe Mcyst-1261 (table). Two other numerically significant methanotroph populations were *Methylocella palustris* and *Methylocapsa acidiphila* (Fig. 3). Oligonucleotide probes M-84 and M-705, which target most known genera of type I methanotrophs, failed to detect any numerically significant population of these organisms in acidic peat. The number of cells targeted by these probes accounted for only 0.1–1% of detectable methanotroph cells.

It should be kept in mind, however, that the values reported above refer to single-time, single peat depth sample measurements. Significant variations in methanotroph community structure and abundance might occur in different sites and depths as well as on a seasonal scale. Our unpublished results show that the total number of methanotrophs detected in *Sphagnum*-dominated wetlands by FISH may be as high as 8×10^7 cells g^{-1} peat. The predominance of type II methanotrophs is characteristic of all acidic (pH 3.5–5.0) boreal wetlands. In contrast, methanotrophic communities in less acidic (pH 5.0–6.0) but colder wetlands of tundra display nearly equal population sizes of type I and type II methanotrophs (Fig. 4). This finding may not be surprising because all known psychrophilic methanotrophs belong to the *Gammaproteobacteria* but are unable to grow at pH below 5.0.

The major disadvantage of using FISH to enumerate methanotrophs is that it can only be applied when the 16S rRNA genes of the target organisms are known. Our recent isolation attempts show that, indeed, not all peat-inhabiting methanotrophs can be detected with the currently available probe set (unpublished data). Therefore, the abundance of methanotrophs in acidic peatlands may have been underestimated.

Stable isotope probing. Stable isotope probing (SIP) is a method which attempts to link the identity of an organism with its biological function under conditions approaching those in situ [49, 50]. Addition of ^{13}C-labeled substrate to an environmental sample results in ^{13}C-labelling of metabolically active bacteria, which use this substrate as a carbon source. DNA of these bacteria becomes heavier and can be separated by CsCl density gradient centrifugation from ^{12}C DNA of bacteria which have not assimilated labeled substrate. The ^{13}C DNA is then used for PCR amplification of phylogenetic (16S rRNA) and functional (*pmoA*, *mmoX* and *mxaF*) genes.

SIP has been applied to identify the functionally active methanotroph populations in a variety of environments [43, 51]. In the study of peat soil [34], 16S rRNA gene sequences amplified from ^{13}C DNA were affiliated with the *Methylocystis/Methylosinus* group, *Methylocella palustris* and *Methylobacter* spp. The majority of *pmoA* sequences recovered from ^{13}C DNA fraction were similar to those of type II methanotrophs, indicating that in the peat soil environment, type II methanotrophs are the dominant bacteria actively assimilating methane [34]. Interestingly, SIP studies of acidic (pH 3.5) forest soil also revealed the predominance of 16S rRNA gene sequences from type II methanotrophs (*Methylocella*, *Methylocapsa* and *Methylocystis*) in a clone library obtained from ^{13}C DNA fraction [52]. The opposite diversity pattern after SIP, when all the *pmoA* sequences obtained were related to those from type I methanotrophs, was revealed in soda lake sediments [53]. These results provide useful information about the ecological niche of different groups of methane oxidizing bacteria: type II methanotrophs are active in low pH environments, while type I methanotrophs are active in high pH environments [51].

Recently, the combination of DNA-SIP, multiple displacement amplification of ^{13}C-labelled DNA and preparation of a fosmid metagenomic library was successfully applied to retrieve relatively large DNA fragments of uncultivated *Methylocystis* in acidic peatlands [41]. Screening of the metagenomic library revealed one fosmid containing methanol dehydrogenase and two fosmids containing 16S rRNA genes from these methanotrophs. The *mxaF*-containing fosmid was shotgun-sequenced, which allowed the assembly of a 14 kb contig containing a cluster of genes known to be involved in bacterial methanol utilization.

Stable-isotope probing has also been combined with RNA extraction (RNA-SIP) because RNA can be a more responsive biomarker than DNA [54, 55]. However, RNA-SIP has not yet been applied to study the functionally active methanotroph populations in acidic wetlands.

One problem in interpreting results of SIP studies involving $^{13}CH_4$ is the recovery of 16S rRNA gene sequences, which are not related to known methanotrophs. In some of these studies, 16S rRNA gene sequences were detected which were related to *Bdellovibrio* sp. and *Cytophaga* sp. or were affiliated with the *Acidobacteria* and *Verrucomicrobia* [34, 40, 52]. Most likely, these clones have resulted from turnover of ^{13}C as a result of predation or from cross-feeding on slime-producing methanotrophs, which are typical of acidic peat environment (see chapter 4).

SIP can also be combined with the analysis of the ^{13}C-labelled phospholipid fatty acids (PLFA) to determine which particular methanotrophic species are responsible for the uptake of $^{13}CH_4$ in environmental samples. This approach has been applied to acidic peat as well [40]. The assimilated ^{13}C was almost entirely found in three PLFAs; 16:1ω7, 18:1ω7 and 18:1ω9.

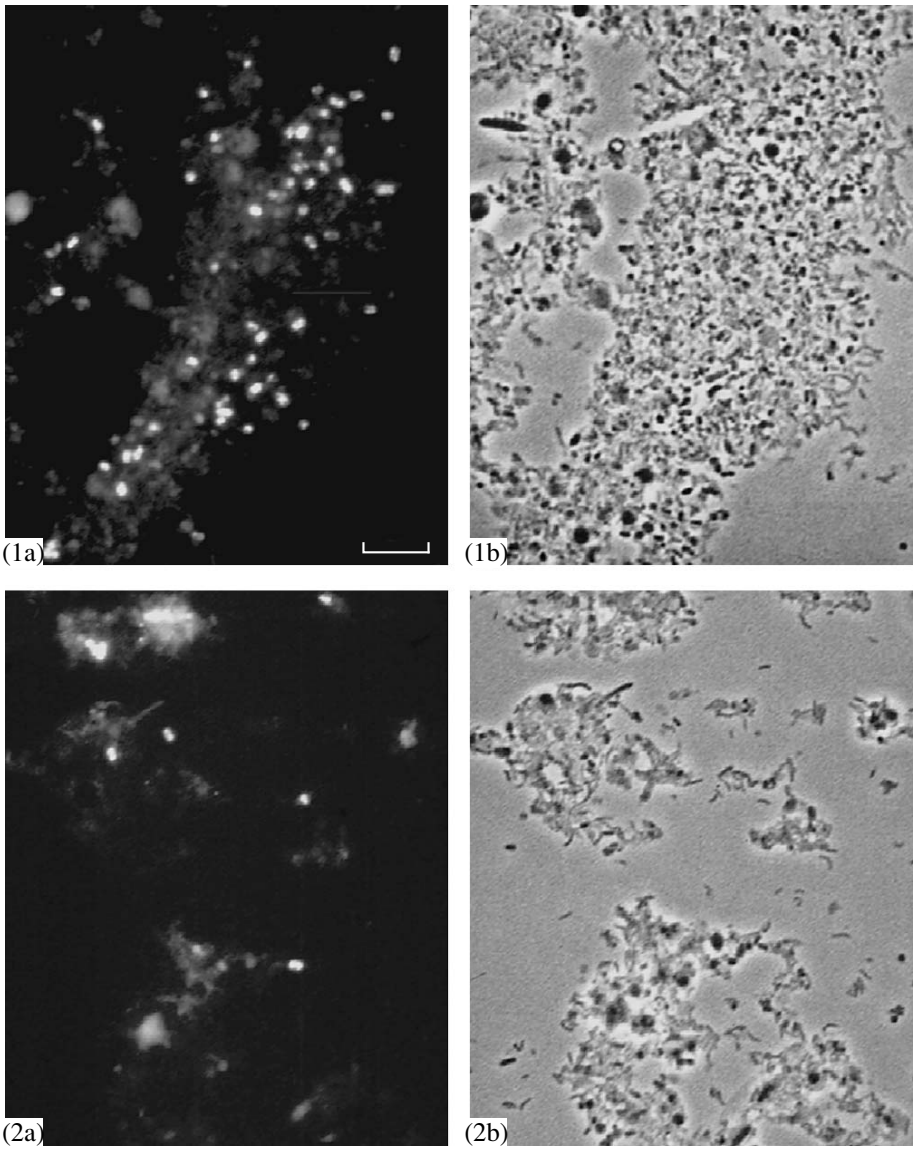

Fig. 3. Specific detection of *Methylocella palustris* (1) and *Methylocapsa acidiphila* (2) in a peat sample by FISH: epifluorescence micrographs of in situ hybridization with probes Mcell-1026 and Mcaps-1032 (a), and the respective phase-contrast images (b). The scale bar (10 μm) applies to all images.

The major ^{13}C-labelled PLFA was 18:1ω7, which is a characteristic PLFA of the acidophilic methanotrophs *Methylocella* and *Methylocapsa* [21, 35, 36]. Peat-inhabiting *Methylocystis* spp. also contain this PLFA [39, 42]. The presence of 16:1ω7 is difficult to interpret since both type I methanotrophs and *Methylocystis heyeri* possess this PLFA [39, 56]. The presence of ^{13}C-labelled 18:1ω9, which is not a major PLFA of any extant methanotrophs, indicated the presence of novel, yet uncultured methanotrophs in acidic peatlands [40].

Microarrays. DNA microarrays are a powerful genomic technology which is used to monitor gene expression under different cell growth conditions, detect specific mutations in DNA sequences and characterize microorganisms in environmental samples. This technique represents an excellent tool for the parallel, high-throughput detection of bacteria and quantitative assessment of their community structure [57, 58].

A microarray targeting the *pmoA* gene was developed for the detection and quantification of methanotrophs [59]. This microarray consisted of 59 oligonucleotide probes designed and validated against the *pmoA* sequences of all known methanotrophs, the *amoA* sequences of the ammonia-oxidizing bacteria and *pmoA*/*amoA* related sequences retrieved from environmental samples. High potential of this *pmoA* microarray was demonstrated in several environmental studies; particularly in analyzing methanotroph communities in landfill cover soils [59, 60]. Later, a method for the mRNA-based application of a *pmoA* microarray

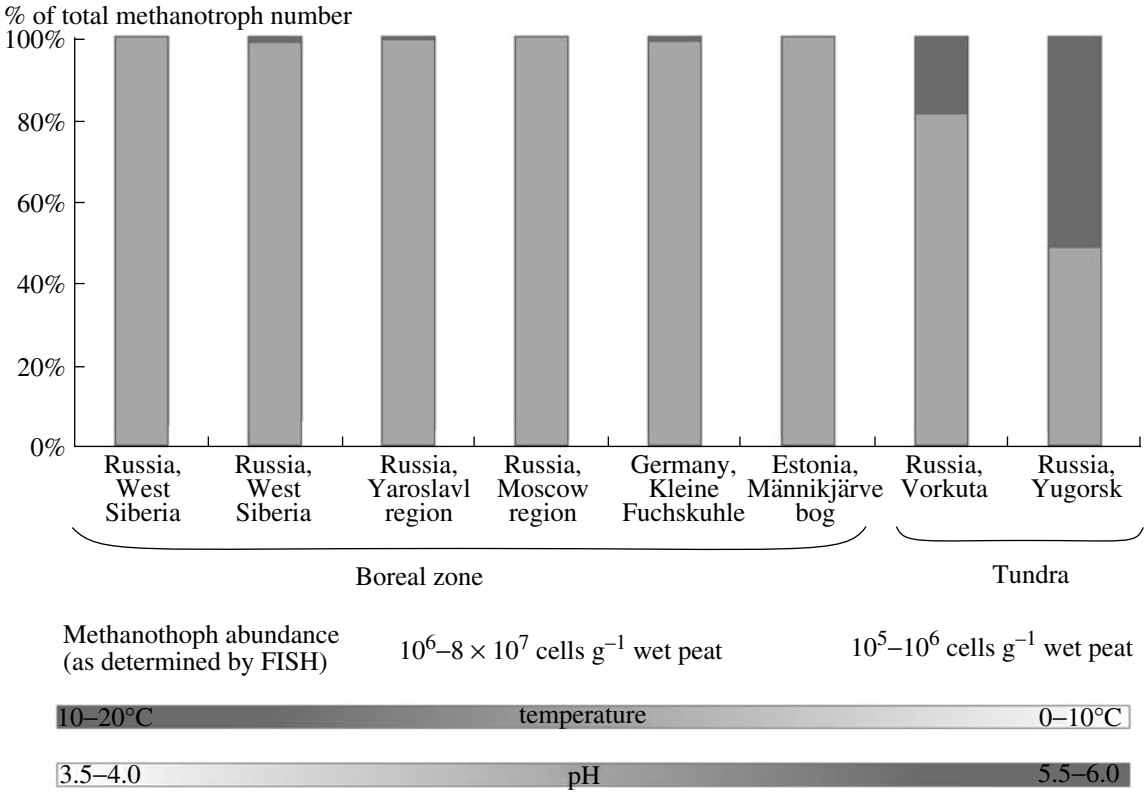

Fig. 4. Proportion of type I (dark grey) and type II (grey) methanotrophs in total methanotroph cell numbers detected by FISH in boreal wetlands and wetlands of tundra.

to detect active methanotroph populations was developed [61] and applied to analyze diversity of the active methanotrophic community in acidic peatlands [40]. Both DNA and RNA were extracted from the peat samples, *pmoA* genes were amplified from DNA and cDNA, which was reverse-transcribed from mRNA, and the diversity of *pmoA* genes and gene transcripts was determined using a *pmoA* diagnostic microarray. Differences in methanotroph community structures were observed between the *Calluna*-covered moorland (pH 4.6) and *Sphagnum*/*Eriophorum*-dominated wetland sites (pH 4.6–4.8). The latter were dominated with *Methylocystis* spp., while *Methylocella* and *Methylocapsa*-related methanotrophs were also present. In *Calluna*-covered moorlands, in addition to *Methylocystis* and *Methylocella*, a unique group of peat-associated type I methanotrophs and a group of uncultivated type II methanotrophs (termed MHP group) were also detected [40]. Thus, the use of a *pmoA* microarray confirmed the predominance of active type II methanotrophs in acidic peatlands.

4. CULTIVATION-BASED STUDIES AND REPRESENTATIVE ORGANISMS

A key to successful isolation of peat-inhabiting methanotrophs is the use of moderately acidic (pH 4.5–5.8) mineral media with a low salt content (0.1–0.5 g l^{-1}) and a low buffering capacity. Conventional mineral media for methanotrophs (containing 1.5–3 g l^{-1} of salt) do not support the growth of these bacteria. The presence/absence of the available nitrogen source in the medium and the conditions of culture aeration (shaking or static incubation) significantly influence methanotroph diversity in the resulting enrichment cultures [62].

Methanotrophic enrichment cultures are commonly obtained by incubation under 30 : 70 methane : air mixture for 6 to 8 weeks at temperatures between 10 and 25°C. As soon as visual turbidity develops, an aliquot is transferred to the fresh medium of the same composition. Isolation of methanotrophic bacteria in pure cultures can be achieved by (1) spread-plating on the surface of a solid medium and (2) multiple serial dilutions in a liquid medium. Usually, a combination of both approaches is required to achieve the culture purity. Most peat-inhabiting methanotrophs do not form colonies on solid media made with agar. Therefore, replacement of agar with gellan gum is recommended for isolation of methanotrophs from low-nutrient environments.

All methanotrophs that have been cultivated from acidic northern wetlands are members of the *Alphaproteobacteria* (Fig. 5). Key traits of these microorganisms are described below.

16S rRNA-targeted oligonucleotide probes that can be used in FISH for detection of methanotrophic bacteria

Probe	Specificity	Probe sequence (5'–3')	Target site (16S rRNA position)[a]	% FA[b]	T (°C)[c]	NaCl, mM[d]	Reference
MG-64	Type I methanotrophs	CCGAAGGCCTRTTACCGTTC	64–83	20	46	900	46
MA-221	*Methylosinus* spp. + some *Methylocystis* spp.	GGACGCGGGCCGATCTTTCG	221–240	35	55	80	46
Mc-1029	*Methylococcus*	CCTGTGTCTTGGCTCCCGAA	2029–1048	10	46	900	46
M-450	Type II methanotrophs	ATCCAGGTACCGTCATTATC	450–470	30	46	112	47
M-84, M-705	Type I methanotrophs	CCACTCGTCAGCGCCCGA CTGGTGGTTCCTTCAGATC	84–103 705–724	20	46	225	47
Mcell-1026	*Methylocella palustris*	GTTCTCGCCACCCGAAGT	1026–1043	0	45–50	900	32
Mcaps-1032	*Methylocapsa acidiphila*	CACCTGTGTCCCTGGCTC	1032–1049	0	45–50	900	32
Mcells-1024	*Methylocella sitvestris*	TCCGGCCAGCCTAACTGA	1024–1041	0	50	900	38
Mcellt-143	*Methylocella tundrae*	TTCCCCGAGTTGTTCCGA	143–160	0	50	900	36
Msint-1268	*Methylosinus trichosporium*	TGGAGATTTGCTCCGGGT	1268–1285	20	46	225	33
Msins-647	*Methylosinus sporium*	TCTCCCGGACTCTAGACC	647–664	30	46	112	33
Mcyst-1432	*Methylocystis* spp.	CGGTTGGCGAAACGCCTT	1432–1449	0	50	900	33
Mcyst-1261	Peat-inhabiting subgroup of *Methylocystis* spp.	TTGCTCGAGGTCGCCCTT	1261–1278	0	50–55	900	33

Notes: [a] Position numbers refer to the *E. coli* 16S rRNA sequence.
[b] Percent formamide in the hybridization buffer.
[c] Temperature of hybridization.
[d] Concentration of sodium chloride in the washing buffer.

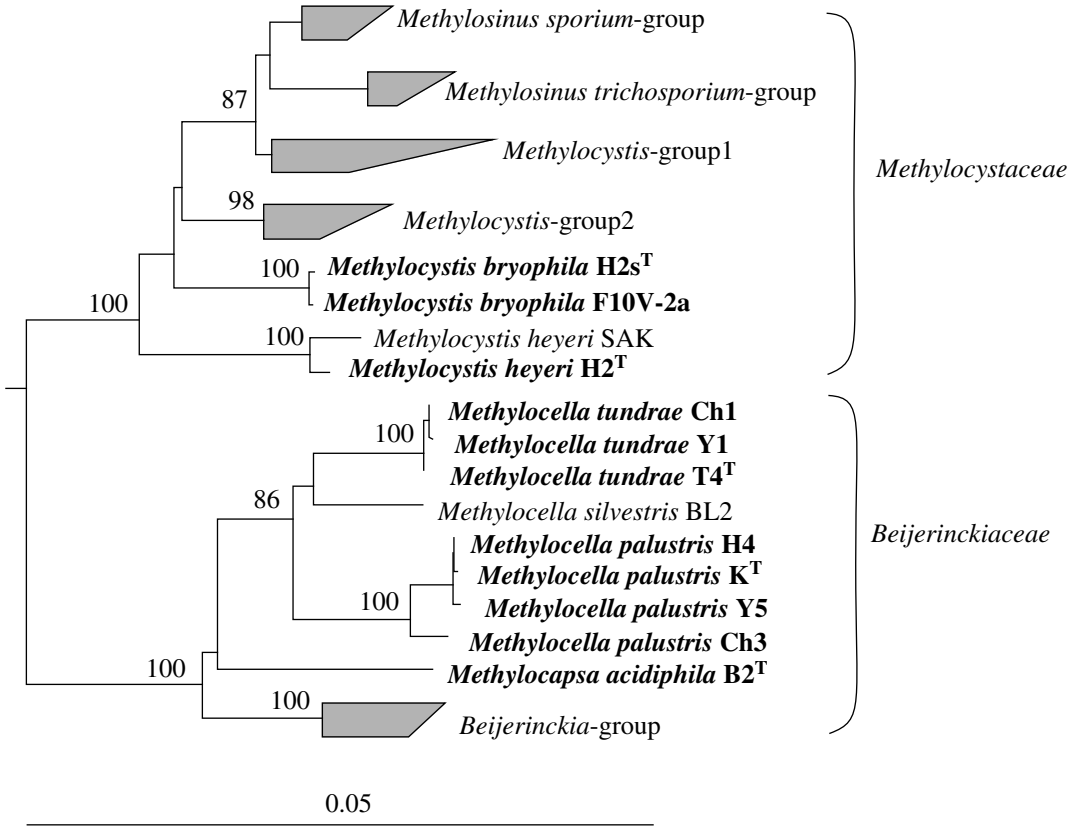

Fig. 5. 16S rRNA-gene based neighbour-joining tree showing the phylogenetic position of methanotrophic bacteria that have been isolated from acidic peat environments (in bold) to other type II methanotrophs and heterotrophic bacteria of the genus *Beijerinckia*. Bar, 0.05 substitutions per nucleotide position.

Genus *Methylocella*. Members of the genus *Methylocella* are widely distributed in acidic and neutral wetlands. The first isolates of these methanotrophs were obtained from acidic peat bogs of West Siberia and European North Russia [31], and described as the novel genus and species *Methylocella palustris* [21]. Later, two more species, *Methylocella silvestris* [48] and *Methylocella tundrae* [36] were isolated from acidic forest and tundra wetlands, respectively.

Based on 16S rRNA gene sequence phylogeny, *Methylocella* species are only moderately related to other alphaproteobacterial methanotrophs in the genera *Methylosinus* and *Methylocystis*, and instead are closely affiliated (96–97% sequence similarity) with chemoorganotrophic bacteria of the genus *Beijerinckia* (Fig. 5). *Methylocella* cells are Gram-negative, nonmotile, polymorphic rods with rounded ends (Fig. 6a). The major distinctive feature of the cells is their highly specific bipolar appearance, which is due to large, highly refractile, intracellular granules of poly-β-hydroxybuturate formed at each pole. The cell ultrastructure is unique among methanotrophs. Regardless of the growth phase or the copper content of the medium, the extensive intracytoplasmic membrane (ICM) structures typical of obligate proteobacterial methanotrophs are absent from *Methylocella* cells. Instead, the cells contain a vesicular membrane system composed of small (40–100 nm in diameter) spherical, ovoid, or tube-shaped vesicles formed by cytoplasmic membrane invaginations. The absence of classic ICM in *Methylocella* coincides well with the absence of particulate, membrane-bound methane monooxygenase that is found in all other methanotrophs. Instead, *Methylocella* uses only a soluble form of this enzyme for methane oxidation. The inferred peptide sequences of the *mmoX* gene in these methanotrophs form a branch distinct from the two other known MmoX sequence groups, i.e. the *Methylosinus/Methylocystis*-like MmoX sequences and the *Methylococcus/Methylomonas* MmoX sequences.

Originally, *Methylocella* spp. were described as aerobic bacteria capable of growth on only the C_1 compounds methane, methanol, methylamine and formate. These substrates are utilized via the serine pathway. It was later observed that members of the *Methylocella* also grew on acetate, pyruvate, succinate, malate and ethanol [38]. Notably, the growth rate and carbon conversion efficiency were higher on multicarbon substrates (for example, on acetate) than on methane, and when both substrates were provided in excess acetate was preferably used and methane oxidation shut down [22, 38]. Therefore, *Methylocella* represents the first

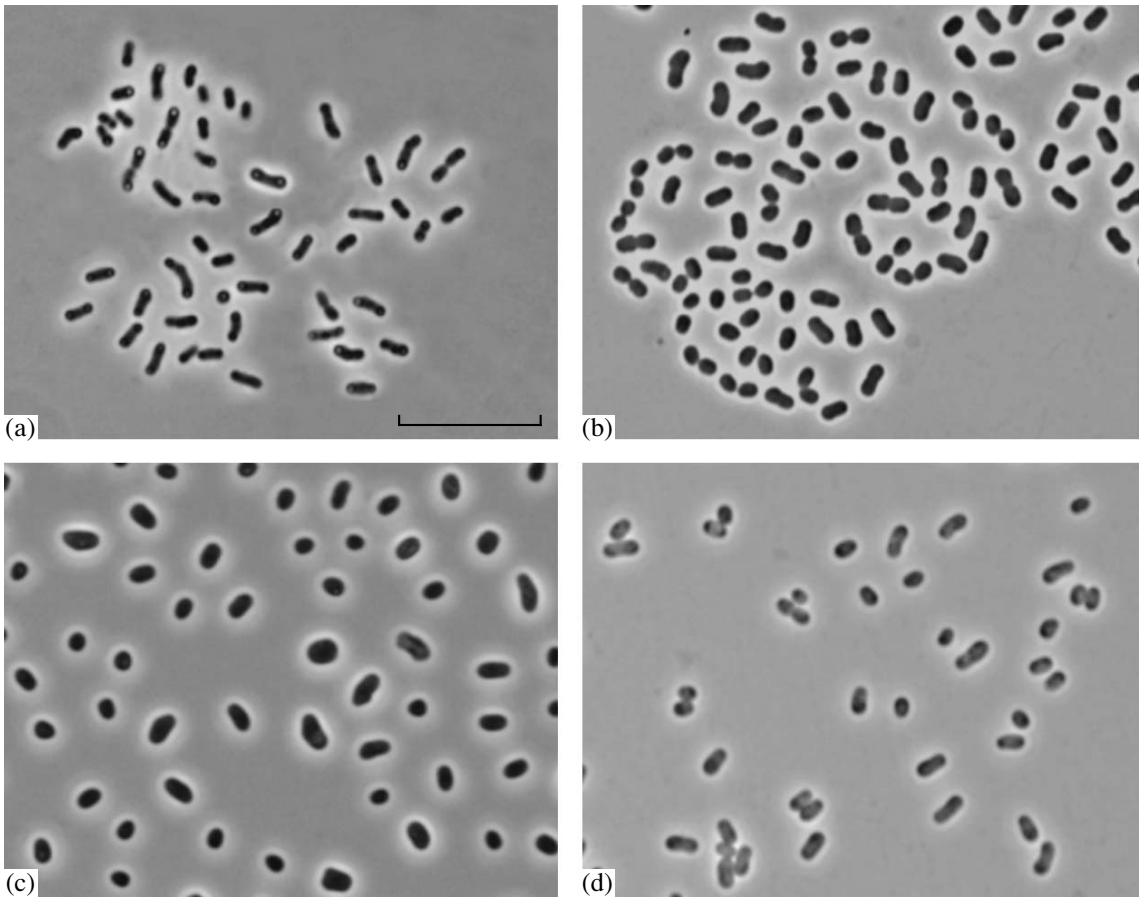

Fig. 6. Phase-contrast micrographs of cells of peat-inhabiting methanotrophic bacteria: *Methylocella palustris* (a), *Methylocapsa acidiphila* (b), *Methylocystis heyeri* (c), and *'Methylocystis bryophila'* (d). The scale bar (10 μm) applies to all images.

fully authenticated facultative methanotroph. All *Methylocella* spp. utilize ammonium salts, nitrates, yeast extract and some amino acids as nitrogen sources. When grown in nitrogen-free media, they are able to fix N_2 via an oxygen-sensitive nitrogenase [63].

The temperature and pH ranges for growth of *Methylocella* spp. are 4–30°C and 4.2–7.5, respectively. *Methylocella tundrae* is slightly more psychrophilic and less acidophilic (growth optimum at 15°C and pH 5.5–6.0) than *Methylocella palustris* (growth optimum at 20°C and pH 5.0–5.5). All members of this genus are highly sensitive to salt stress and prefer diluted media with a low salt content (0.2–0.5 g l^{-1}). The major fatty acid in all members of the *Methylocella* is 18:1ω7*c* (60–82% of the total fatty acids). Cells of *Methylocella tundrae* contain also significant amounts of cy19:0ω8*c* (8–13.5% of the total fatty acids). The major phospholipids are phosphatidylmethylethanolamines. The G+C content of the DNA is 60.0–63.3 mol %.

Genus *Methylocapsa*. *Methylocapsa acidiphila* B2 was isolated from *Sphagnum-Carex* peat of Bakchar bog, West Siberia (Fig. 6b) [35]. This moderately acidophilic methanotroph is phylogenetically closely related (96–97% 16S rRNA gene sequence identity) to *Methylocella* spp. However, *Methylocapsa* is an obligate methanotroph with a pMMO enzyme and a well-developed system of ICM, which are packed in parallel on only one side of the cell membrane. This pattern of ICM arrangement is different from the ICM of both type I and type II methanotrophs, and was termed type III. In contrast to *Methylocella*, *Methylocapsa* does not express sMMO.

The inferred peptide sequence of *pmoA* of *Methylocapsa acidiphila* B2 belongs to a novel PmoA lineage, which is only distantly related to the PmoA sequence cluster of type II methanotrophs (70–72% sequence identity) [64]. This novel PmoA lineage encompasses a number of sequences detected via cultivation-independent methods in several acidic upland soils showing atmospheric methane consumption [65, 66]. So far, *Methylocapsa acidiphila* B2 represented the only cultivated representative of this PmoA lineage.

Methylocapsa uses methane and methanol as sole sources of carbon and energy and utilizes the serine pathway for carbon assimilation. Methanol supports growth only when used at concentrations below 0.05% (v/v). Multicarbon substrates are not utilized. Nitrogen

sources are ammonium salts, nitrates, yeast extract and N_2. In contrast to all other known dinitrogen-fixing methanotrophs (*Methylosinus*, *Methylocystis*, *Methylocella*, *Methylococcus* and *Methylomonas*), *Methylocapsa acidiphila* B2 is capable of exponential growth in liquid nitrogen-free media under fully aerobic conditions [63]. This growth capability of *Methylocapsa acidiphila* is very similar to that of *Beijerinckia*, which was one of the first bacteria described as being capable of fixing N_2 effectively. Interestingly, the NifH fragment from *Methylocapsa acidiphila* B2 is nearly identical (98.0–98.5% sequence identity) to NifH fragments from *Beijerinckia* spp., but shows only 90.8% sequence identity to NifH from *Methylocella* spp. The mechanisms of dinitrogenase protection from oxygen in this acidophilic methanotroph remain unknown.

The temperature and pH ranges for growth of *Methylocapsa acidiphila* are 10–30°C and 4.2–7.2, respectively. Similar to *Methylocella* spp., *Methylocapsa* is highly sensitive to salt stress and prefers dilute media of low salt content. The major fatty acid in *Methylocapsa acidiphila* is 18:1ω7*c* (78% of the total fatty acids) and the major phospholipids are phosphatidylglycerols. The G+C content of the DNA is 63.1 mol %.

Peat-inhabiting members of the genus *Methylocystis*. In acidic peatlands, *Methylocystis*-like methanotrophs are one of the numerically dominant and metabolically active populations. Exact taxonomic affiliation and phenotypic characteristics of these peat-inhabiting methanotrophs remained unclear for a long time. Currently, two representatives of these microorganisms are available in cultures—*Methylocystis heyeri* [39] and '*Methylocystis bryophila*' [42].

Two representative strains of *Methylocystis heyeri*, H2 and SAK, were isolated from an acidic (pH 4.3) *Sphagnum* peat bog lake Teufelssee, Germany, and an acidic (pH 4.2) tropical forest soil, Thailand, respectively. Cells of these methanotrophs non-motile, straight or curved rods that are covered by large polysaccharide capsules (Fig. 6c) and contain an intracytoplasmic membrane system typical of type II methanotrophs. They possess both a particulate and a soluble methane monooxygenase. Growth substrates are methane and methanol, which are utilized via the serine pathway. Multicarbon substrates are not used. Nitrogen sources are ammonium salts, nitrates, yeast extract, some amino acids and N_2. The temperature and pH ranges for growth of *Methylocystis heyeri* are 5–30°C and 4.4–7.5, respectively.

The most unique characteristic of *Methylocystis heyeri* is the phospholipid fatty acid profile. In addition to the signature fatty acid of type II methanotrophs (18:1ω8*c*), cells also contain large amounts of what was previously considered a signature fatty acid of type I methanotrophs, 16:1ω8*c*. Interestingly, significant amounts of 16:1ω8*c* fatty acid were detected in extracts made from acidic peat in early studies on methanotrophy in *Sphagnum*-dominated wetlands [13, 67]. This was interpreted to mean that type I methanotrophs were abundant. By contrast, *in situ* hybridization with methanotroph-specific oligonucleotide probes detected only relatively small populations of type I methanotrophs in acidic peat [32, 33]. Analysis of PLFA profiles in *Methylocystis heyeri* offers a possible explanation for this controversy and suggests that there are severe difficulties with the use of 16:1ω8*c* as a specific biomarker for type I methanotrophs.

'*Methylocystis bryophila*' is also represented by the two strains, F10V2a and H2s, which were isolated from the surface sediment of the dystrophic peat bog lake Fuchskuhle and *Sphagnum* peat on the bank of the bog lake Teufelssee, northeastern Germany, respectively [42, 69], but only strain H2s (Fig. 6d) was characterized in detail. This is mesophilic and mildly acidophilic methanotroph, which grows at 8–35°C and pH 4.5–7.5 with the optimum at 25–30°C and pH 6.0–6.5. Strain H2s possesses both a particulate and a soluble form of methane monooxygenase and a well-developed ICM system. It demonstrates a clear preference for growth on methane but is able to grow slowly on acetate in the absence of methane. The efficiency of carbon conversion to cell material for strain H2s is significantly higher on methane (47.0 ± 6.0%) than on acetate (18.0 ± 1.4%). Yet, this methanotroph is able to survive multiple transfers on a medium with acetate as the only growth substrate. Interestingly, in cells grown for several transfers on acetate, ICM are maintained, although in a reduced form, and mRNA transcripts of particulate MMO are detectable. Therefore, this isolate is the first example of a pMMO-possessing facultative methanotrophs. The major PLFAs are C18:1ω8*c* and C18:1ω7*c*. Growth on acetate leads to a major shift in the phospholipid fatty acid composition.

FISH-based studies revealed that strain H2s-like cells comprise 17–58% of all type II methanotrophs detected in *Sphagnum*-dominated wetlands of various geographic locations, e.g. Northeastern Germany, European North Russia and West Siberia. Phylogenetic analysis based on fragments of the *pmoA* gene also confirmed that strain H2s belongs to a large cluster of environmental *pmoA* sequences that have been retrieved from acidic boreal peatlands [29, 41, 68] and acidic forest soil [52]. Therefore, strain H2s-like organisms seem to represent a numerically and ecologically important methanotroph population in acidic terrestrial ecosystems of the Northern hemisphere.

Novel metabolic traits in peat-inhabiting methanotrophs. Examination of these novel microorganisms from acidic wetlands revealed several previously unknown traits and metabolic capabilities in methanotrophic bacteria. Until recently, all known methanotrophs were thought to share two common features: (i) possession of pMMO and (ii) inability to utilize multicarbon compounds for growth. Physiological and genomic studies on *Methylocella* spp. demonstrated that these methanotrophs are an exception to both of the

"paradigms" [21, 22, 38]. They use only sMMO for methane oxidation, which is repressed if an alternative multicarbon growth substrate is available.

In methanotrophs that possess both pMMO and sMMO, sMMO is expressed only under low copper-to-biomass ratios, while pMMO is expressed under high copper-to biomass ratios [23, 70]. Interestingly, this regulation does not exist in *Methylocella* spp. As shown by Theisen et al. [22], sMMO was expressed in cells of *Methylocella silvestris* $BL2^T$ grown in both high-copper (1 μM) and low-copper (5 nM) media. This means that the mechanism of sMMO activity regulation in *Methylocella* is different from that observed in other known methanotrophs.

The peat-inhabiting species *'Methylocystis bryophila'* is the first example of a pMMO-possessing facultative methanotroph. In contrast to sMMO-possessing *Methylocella* spp., these bacteria show clear preference for growth on methane. However, they also have the ability to grow slowly on acetate when methane is unavailable. This previously unknown metabolic flexibility in *Methylocystis* provides an excellent explanation for the exceptionally wide distribution and good survival of these methanotrophs in diverse terrestrial environments, such as wetlands, soils, sediments and rice paddies. The existence of other pMMO-possessing methanotrophs capable of growth on acetate remains to be elucidated.

5. METHANOTROPHS AWAITING ISOLATION

Several other methanotroph populations have been detected in acidic peat by molecular approaches, but have so far eluded isolation. These "missing" organisms are listed below.

Peat-inhabiting type I methanotrophs. According to the results of FISH-based studies, members of the *Methylococcaceae* do not represent a numerically significant methanotroph population in acidic wetlands [32, 33]. However, type I methanotrophs can be detected in peat by hybridization with 16S rRNA-targeted fluorescent probes and by PCR-based recovery of the 16S rRNA, *pmoA* or *mmoX* gene fragments. Stable isotope probing with $^{13}CH_4$ of an acidic peat in the UK identified *Methylobacter*-like bacteria among metabolically active methanotroph populations [34]. *Methylococcus*-like *pmoA* sequences were recovered from a Finnish raised mire [68], and *Methylobacter* were detected using a *pmoA*-based microarray analysis of heather moorlands and *Sphagnum/Eriophorum*-covered UK peatlands [40]. Several type I methanotroph *mmoX* sequences were also retrieved from *Sphagnum/Eriophorum* peatlands [40]. Attempts to isolate these acidophilic/acidotolerant members of the *Methylococcaceae* have so far been unsuccessful.

"Symbiotic" methanotrophs associated with *Sphagnum* plants. The first microscopic observation of methanotrophic bacteria within hyaline cells (water-filled, dead, porous cells) of *Sphagnum* mosses was reported in 1999 [71]. Later, the presence of numerous alphaproteobacterial methanotrophs in the hyaline cells of the outer stem cortex and on the surface of stem leaves of *Sphagnum cuspidatum* was demonstrated by molecular approaches [37]. Experiments with ^{13}C-methane showed its rapid in situ oxidation by *Sphagnum*-associated methanotrophs to CO_2, which was subsequently fixed by *Sphagnum*, as shown by incorporation of ^{13}C-label into plant sterols. Molecular identification of these methanotrophs occurring as dense, geometric clusters of cells, showed their distant affiliation to acidophilic methanotrophs of the genera *Methylocella* and *Methylocapsa* (93% 16S rRNA gene sequence identity). Several other, similar 16S rRNA gene sequences (peat bog clones B7, B26, B29, B49 and B83) were later retrieved from acidic *Sphagnum* peat bog in West Siberia [72] as well as from cellulolytic peat enrichment cultures, which were incubated without methane (enrichment clones A26 and B35) (unpublished data). These sequences form a common cluster, which is distinct from the *Methylocystaceae* and the *Beijerinckiaceae* (Fig. 7). The biology of methanotrophs within this cluster remains obscure. According to the images obtained by transmission electron microscopy, these methanotrophs do not contain ICM [37]. Therefore, it is very likely that they are facultatively methanotrophic bacteria similar to *Methylocella* spp.

Peat-inhabiting *Verrucomicrobia*. The occurrence of methanotrophic *Verrucomicrobia* in acidic northern wetlands is a highly intriguing question. So far, methanotrophic *Verrucomicrobia* have been found only in geothermal habitats [16]. They are extreme acidophiles with a growth optimum at pH 2.0–3.5. As was shown in early studies, methanotrophic activity in acidic peat peaks at pH 5–5.5 and declines at pH below 3.0 [73, 74]. This, of course, argues against the occurrence of '*Methylacidiphilum*'-like *Verrucomicrobia* in a *Sphagnum*-derived peat. None of the currently published results of $^{13}CH_4$-SIP experiments targeting DNA of methanotrophs in acidic peatlands support the notion for a wider distribution of methanotrophic *Verrucomicrobia*. However, *Verrucomicrobia*-related 16S rRNA gene sequences are abundantly represented in clone libraries obtained from *Sphagnum* peat [72] and we also retrieved these sequences from methanotrophic enrichment cultures isolated from acidic peat (unpublished data). Therefore, further studies should address the ecological diversity and distribution of these newly discovered methanotrophs.

6. CONCLUSIONS AND FUTURE PROSPECTS

It is now evident that northern wetlands accommodate two physiologically distinct types of methanotrophs; specialist populations that grow only on methane (and other C_1 compounds), and generalist populations

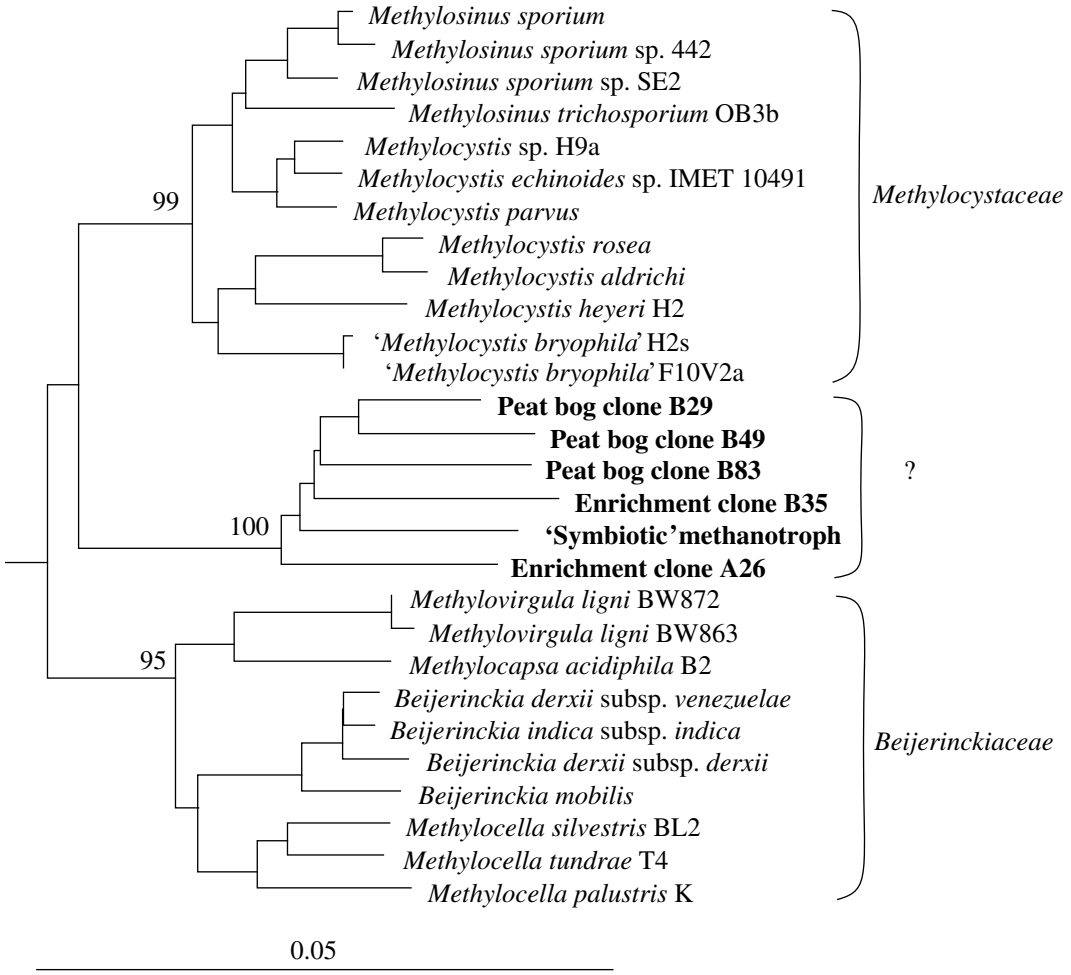

Fig. 7. 16S rRNA-gene based neighbour-joining tree showing the phylogenetic position of putative "symbiotic" methanotrophs in relation to other representatives of the families *Methylocystaceae* and *Beijerinckiaceae*. Bar, 0.05 substitutions per nucleotide position.

with the ability to metabolize several multicarbon compounds besides methane. One of these compounds is acetate, a major end product of anaerobic metabolism in wetlands. To make things even more complex, facultative methanotrophs are also not uniform with regard to their substrate preferences. One group is represented by *Methylocella*-like, sMMO-possessing methanotrophs with the preference for growth on multicarbon compounds. Our recent findings indicate that their diversity is not restricted to representatives of the genus *Methylocella*. Another group of facultative methanotrophs is represented by *Methylocystis*-like, pMMO-possessing bacteria with a clear preference for growth on methane but with the ability to grow slowly or to survive on acetate as well. Therefore, the previous basic assumption that methanotroph populations are energetically limited only by the supply of methane is in error. Further studies are needed to address the exact role of facultative methane oxidizers in reducing methane emissions from wetlands and environmental changes that switch facultative methanotrophs from methane to acetate consumption.

Peat-inhabiting methanotrophs represent interesting objects for comparative genomic analysis. Two of these bacteria, *Methylocella* and *Methylocapsa*, are the subject of genome sequencing project, which is now in progress at the Joint Genome Institute of the US Department of Energy. Comparing genomes of these phylogenetically close but phenotypically distinct methanotrophs may shed some light into the genetic and metabolic tradeoffs required for an obligately methanotrophic lifestyle compared to the facultatively methanotrophic lifestyle. *'Methylocystis bryophila'*, the first pMMO-possessing facultative methanotroph, is also an exciting object for the genomic and proteomic studies.

Finally, further effort to cultivate "missing methanotrophs" from acidic wetlands is required to fully understand the biology and physiology of the microorganisms responsible for the uptake of methane in these vast terrestrial environments.

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