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Diversity of Aerobic Methanotrophic Bacteria in a Permafrost Active Layer Soil of the Lena Delta, Siberia

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Abstract With this study, we present first data on the diversity of aerobic methanotrophic bacteria (MOB) in an Arctic permafrost active layer soil of the Lena Delta, Siberia. Applying denaturing gradient gel electrophoresis and cloning of 16S ribosomal ribonucleic acid (rRNA) and *pmoA* gene fragments of active layer samples, we found a general restriction of the methanotrophic diversity to sequences closely related to the genera *Methylobacter* and *Methylosarcina*, both type I MOB. In contrast, we revealed a distinct species-level diversity. Based on phylogenetic analysis of the 16S rRNA gene, two new clusters of MOB specific for the permafrost active layer soil of this study were found. In total, 8 out of 13 operational taxonomic units detected belong to these clusters. Members of these clusters were closely related to *Methylobacter psychrophilus* and *Methylobacter tundripaludum*, both isolated from Arctic environments. A dominance of MOB closely related to *M. psychrophilus* and *M. tundripaludum* was confirmed by an additional *pmoA* gene analysis. We used diversity indices such as the Shannon diversity index or the Chao1 richness estimator in order to compare the MOB community near the surface and near the permafrost table. We determined a similar diversity of the MOB community in both depths and suggest that it is not influenced by the extreme physical and geochemical gradients in the active layer.

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Introduction

Aerobic methanotrophic bacteria (MOB) of the phylum *Proteobacteria* primarily contribute to the consumption of methane in terrestrial environments. The microbial conversion of methane into carbon dioxide was estimated to account for 30% to 90% in flooded rice fields [1, 2], for 13% to 38% of the methane produced in temperate and subarctic peat soils [13], and for 15% to more than 90% of the diffusive methane flux in wetlands [36, 47]. The group of MOB comprises the three families *Methylococcaceae*, *Methylocystaceae*, and *Beijerinckiaceae* [5, 9–12]. The only exception is *Crenothrix polyspora*, a filamentous, sheathed microorganism recently discovered to be methanotrophic [52]. Members of the *Methylococcaceae* are termed type I MOB and belong to the γ-subdivision of the *Proteobacteria* phylum. Members of the *Methylocystaceae* and *Beijerinckiaceae* are termed type II MOB and belong to the α-subdivision of the *Proteobacteria* phylum [10–21]. The diversity and composition of MOB have been investigated in several environments such as freshwater sediments [8, 42], landfill soils [59], rice field soils [22, 24], habitats with only atmospheric methane concentrations [29, 33, 35], and peat bogs with very low pH values [39, 40].

Northern wetlands and tundra contribute about 20% to the global natural methane emission annually [6, 7, 18]. Given that the global warming potential of methane on a molecular basis and a time horizon of 100 years is 23-fold that of carbon dioxide [27], the ecology of the MOB community as the major sink for methane in these environments is of importance. Methanotrophic communities were found to be abundant and active also in cold environments such as, for example, northern peat lands, eastern Antarctica, and Fennoscandian deep ground waters

[53]. It was reported that MOB are viable in deep Siberian permafrost sediments with ages of 1,000–100,000 years as well [32]. In addition, based on the temperature response of potential methane oxidation rates, it was recently shown that methane oxidizing bacteria are well adapted to the temperature regime in permafrost active layer soils of the Lena Delta [37]. However, our knowledge on MOB from high-latitude environments in terms of diversity and composition remains very poor [56].

With this study, we present first data on the methanotrophic diversity (richness and evenness) in Arctic tundra soils of the Lena Delta with steep gradients of temperature and methane. In addition to the 16S ribosomal ribonucleic acid (rRNA) gene, we analyzed the phylogeny of the particulate methane monooxygenase gene (*pmoA*), known as a functional marker for MOB [40]. With this study, we will show to what extent the methanotrophic diversity is influenced by the harsh environmental conditions in Siberian permafrost active layer soils.

Materials and Methods

Study Site

The study site is located in the eastern part of Samoylov Island (N 72°22′, E 126°28′) in the Lena Delta, Siberia. For a detailed description of the geomorphology of the Lena Delta, refer to Schwamborn et al. [50]. During the expedition LENA 2005 (7th of July to 1st of September), active layer cores (ø 56 mm) of a low-centered polygon were sampled. The cores were frozen immediately after sampling and were kept frozen until further processing. For this study, we used core number 33, sampled on the 25th of August, 2005, at the rim of the polygon. In the laboratory, the core was sectioned under sterile conditions, and samples of depths 6–11 (near surface) and 20–28 cm (near permafrost table) were selected for molecular analysis. The selection of these samples was according to a previously observed shift in the temperature optima of potential methane oxidation rates between the near surface and the near permafrost table within the active layer of the polygon rim [37].

Soil and Pore Water Analysis

Vertical profiles of soil CH_4 concentrations were obtained from the elevated rim of the polygon through extraction of CH_4 from soil pore water by injection of 5 ml water into saturated NaCl solution, shaking the solution and subsequently analyzing the CH_4 headspace concentration with gas chromatography. Soil temperature measurements (Greisinger GTH 100/2 equipped with Ni–Cr–Ni temperature sensor) were carried out in 5-cm increments from 0 to 38 cm soil depth before core sampling. The total carbon and total nitrogen (TN) contents were determined with an automatic element analyzer (Elementar VARIO EL III). The total organic carbon (TOC) content was measured on corresponding samples after HCl (10%) acid digestion to remove the carbonate on the same analyzer (Elementar VARIO EL III). The pH values were determined with the MultiLab 540 (WTW, Germany) in pore water, which was extracted by applying Rhizons into undisturbed active layer samples according to the method of Seeberg-Elverfeldt et al. [51]. Cell counts of MOB were obtained based on fluorescence in situ hybridization with the MOB-specific probes Mg705/Mg84 (type I MOB) and Ma450 (type II MOB) [15] according to a protocol described recently [37].

Extraction of Total DNA

Prior to the deoxyribonucleic acid (DNA) extraction, the core sections were thoroughly homogenized. Afterwards, four subsamples of each depth were used for extraction. Total genomic DNA was extracted with the UltraClean™ Soil and the PowerSoil™ DNA Isolation Kit (Mo Bio Laboratories, USA) following to the manufacture's protocol. Both kits combine heat, detergents, and mechanical force against beads to lyse microbial cells. The released DNA is then bound to a silica spin filter and finally purified. The PowerSoil™ DNA Isolation Kit is intended for use with environmental samples containing a high humic acid content. The size of the genomic DNA was checked by electrophoresis on a 1% agarose gel against a Lamda *Eco*RI/*Hin*dIII marker (ABgene, UK) with SYBR gold staining.

PCR Amplification

Polymerase chain reaction (PCR) amplification reactions were performed with a thermal cycler (iCycler, Bio-Rad, USA). PCR reaction mixes (50 μl for denaturing gradient gel electrophoresis [DGGE], 25 μl for clone libraries) contained 1× PCR reaction buffer, 0.2 μM primer, 0.25 μM deoxyribonucleoside triphosphates, 1.25 U MasterTaq Polymerase (Eppendorf, Germany), and 5–30 ng template (in a 1:10 dilution). All reactions were optimized against $MgCl_2$ and PCR enhancer solution (Eppendorf, Germany). PCR conditions were as follows: 94°C for 3 min (initial denaturation), followed by 30 cycles (DGGE analysis) or 20 cycles (clone libraries) with 30 s at 94°C, 1 min at 56°C, and 3 min at 72°C, and a final elongation at 72°C for 10 min (DGGE analysis) or 60°min (clone libraries). MOB-specific 16S rRNA gene sequences were amplified using the primer combinations MethT1b F/MethT1b R (type I MOB) and 27 F/MethT2 R (type II MOB). For the

amplification of the *pmoA* gene, a nested PCR approach (A189 F/A682 R followed by A189 F/mb661 R/A650 R) as suggested by Horz et al. [29] was compared with a direct PCR approach (A189 F/mb661 R/A650 R). Because the nested approach did not improve DGGE or cloning efficiency but was likely to increase PCR bias and contamination by *amoA* gene (encoding subunit A of the ammonia monooxygenase) sequences, the direct approach was chosen for the analysis. Primer details and references are listed in Table 1. PCR products were checked by electrophoresis on a 2% agarose gel against a 100-bp marker (MoBiTec, Germany) with SYBR gold staining. For DGGE, PCR products were purified with a QIAquick PCR Purification Kit (Qiagen, Germany). For clone libraries, PCR products in plates (96-well plates, ABgene) were purified by centrifugation (980×*g* at 4°C) through Multi Screen 96-well plates (Millipore, USA) on a column of Sephadex™ G-50 Superfine powder (GE Healthcare Bio-Science, Sweden).

Denaturing Gradient Gel Electrophoresis

PCR products were separated on an 8% polyacrylamide gel in 1×Tris–acetate–ethylenediamine tetraacetic acid buffer using a D-Code System (Bio-Rad). The denaturing gradient (100% denaturant consisted of 7 M urea and of 40%, *v*/*v*, deionized formamide) ranged from 30% to 60% and from 40% to 70%, respectively. Gradient gel electrophoresis ran constantly at 100 V for 14 h at 60°C. The gels were stained for 30 min with SYBR gold and visualized with a GeneFlash system (Syngene, UK). Distinct bands were excised with a sterile scalpel, eluted overnight at 4°C, and reamplified with primers without GC-clamp in 20–25 cycles. Products were purified as described and sequenced by MWG (Germany).

Construction of Clone Libraries and Sequencing

The purified PCR products were ligated into the linear Plasmid Vector pCR4 supplied with the TOPO TA Cloning^{R} Kit for Sequencing (Invitrogen, Germany) and subsequently transformed into chemically competent *E. coli* cells via heat shock following the manufacture's protocol. Cells were incubated overnight at 37°C on agar plates containing 0.05% Ampicillin. Only cells containing a Vector with the insert were competent to grow with ampicillin. Colonies of these cells were screened for correct size of the insert and directly sequenced. Sequencing was performed with the BigDye Terminator cycle sequencing kit, version 3.1 (Applied Biosystems, Germany) with vector primers.

Sequence Analysis

Sequences of the 16S rRNA gene were automatically and manually edited with Sequencing Analysis 5.2 (Applied Biosystems) and checked for chimeras with Bellerophon [30] and with the Chimera-Check of the Ribosomal Database Project (Michigan State University). Contigs were assembled with Sequencher 4.7 (Gene Codes, USA). Sequences were imported into the ARB 16S rRNA database (www.arb-home.de) and phylogenetically analyzed. Rarefaction analysis and estimation of diversity indices were performed with DOTUR [49].

Previous to an analysis of the achieved *pmoA* gene sequences, a database of *pmoA* gene sequences was created using CLC Free workbench software (http://www.clcbio.com). This database contains all translated *pmoA* gene sequences of good quality (approximately 1,000 sequences) available in GenBank (http://www.ncbi.nlm.nih.gov/). The translated *pmoA* gene sequences were aligned using the

Table 1 Methanotroph specific primers used in this study

Target gene	*E. coli* position[a] fragment length (bp)[b]	Primer[c]	Sequence[f]	Annealing (°C)	Reference
16S rRNA	84–102	MethT1bF[d]	5′-CCT TCG GGM GCY GAC GAGT-3′	56	[59]
	988–1,006	MethT1bR	5′-GAT TCY MTG SAT GTC AAGG-3′		[59]
	27–46	27F[d,e]	5′-GAG TTT GAT CMT GGC TCA G-3′		[34]
	997–1017	MethT2R[e]	5′-CAT CTC TGR CSA YCA TAC CGG-3′		[59]
pmoA		A189 F[d]	5′-GGN GAC TGG GAC TTC TGG-3′	56	[26]
	493	A682 R	5′-GAASGCNGAGAAGAASGC-3		[26]
	472	Mb661 R	5′-CCG GMG CAA CGT CYTTACCC-3′		[8]
	461	A650 R	5′-ACG TCC TTA CCG AAG GT-3′		[3]

[a] Refers to 16S rRNA gene primers
[b] Refers to *pmoA* gene primers
[c] Primer combinations are given in "Materials and Methods"
[d] GC clamp for DGGE: 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′
[e] Increasing annealing time: 45, 60, and 90 s according to Wise et al. [59]
[f] *N* Bases A, C, T, or G, *M* bases A or C, *Y* bases C or Z, *S* bases G or C

MUSCLE algorithm [14]. Database and alignment were imported into the ARB program. The *pmoA* sequences obtained in this study were translated with CLC Free workbench, imported into the newly created *pmoA* gene database, manually aligned, and phylogenetically analyzed.

Nucleotide and Protein Sequence Accession Numbers

The 16S rRNA and *pmoA* gene sequences recovered in this study and used for phylogenetic presentation have been deposited in the European Molecular Biology Laboratory, GenBank, and DNA Data Bank of Japan nucleotide sequence databases under the accession numbers EU124838–56 (16S rRNA gene sequences) and EU124857–EU124864 and EU135968 (*pmoA* gene sequences).

Results

Soil and Pore Water Characteristics

The soil of the polygon rim was characterized by a soil texture of silty and loamy sand, a low water level leading to oxic conditions in the top soil compared to the bottom, and a limited organic matter accumulation. This was reflected by relatively low contents of TOC (1.2–3.0%) and TN (approx. 0.1%). During the summer months, the active layer, furthermore, showed distinct gradients in temperature, which decreased from the top to the bottom (8.8–0.9°C) and methane concentration, which increased with soil depth (52.1–176.5 nmol g^{-1}). The pH was slightly acidic to almost neutral (6.1–6.5). The values of TOC, TN, pH, methane, and temperature are summarized in Table 2. Cell counts of type I MOB varied between 1.6×10^7 ($\pm4.7\times10^6$) cells g^{-1} soil near the surface and 7.5×10^6 ($\pm1\times10^6$) cells g^{-1} soil near the permafrost table. In contrast, cell counts of type II MOB were below the detection limit that was at 2.4×10^4 cells g^{-1} soil according to the high dilution necessary to obtain low autofluorescence of the mineral and organic soil matter.

Diversity of Aerobic Methanotrophic Bacteria Based on the 16S rRNA Gene

The amplification of the 16S rRNA gene using methanotroph-specific primers revealed only sequences of the type I MOB but not of the type II MOB. The DGGE profiles of these 16S rRNA gene sequences of both depths showed only a few bands (Fig. 1a). Two distinct bands of the near-surface samples and three of the near-permafrost table samples were excised and sequenced. In Table 3, the sequences are assigned to the according bands in the DGGE gel, and their phylogenetic relatedness is summarized. Briefly, both depths were represented by members of the genera *Methylobacter* and *Methylosarcina* belonging to the γ-subdivision of the *Proteobacteria* phylum (type I methanotrophs). In both depths, the closest cultured relatives of the obtained sequences were *Methylobacter psychrophilus*, *Methylosarcina quisquiliarum*, and *Methylosarcina fibrata*. In addition, one sequence of the near permafrost table was most closely related to *Methylobacter tundripaludum*.

For clone library analysis, 55 sequences were used in total, 35 belonging to the near-surface samples and 20 belonging to the near-permafrost table samples. In total, 13 operational taxonomic units (OTUs) were identified (≥98% sequence similarity). Except for one OTU grouping within the *Methylosarcina* genus, they were all closely related to the *Methylobacter* genus. Again, no type II methanotrophs could be detected. Considering both depths separately, nine OTUs were detected near the surface and eight near the permafrost table. Of all OTUs, five were restricted to the near-surface and four to the near-permafrost table samples. The phylogenetic affiliation of selected sequences representing the different OTUs is shown in Fig. 2. According to this, the OTUs group in four clusters (clusters I to IV). Clusters I and II are specific for the active layer studied here and show a distinct microdiversity. These clusters consist of three and five OTUs, respectively, corresponding to 41 out of 55 sequences in total. Sequences of these clusters are greater than 3% and less than 10% different

Table 2 Selected soil properties of the elevated polygonal rim on Samoylov Island, Lena Delta (values of methane concentrations and temperatures were obtained in the field in July, 2005)

Depth (cm)	pH	TOC (%)	TN (%)	CH_4 (nmol g^{-1})	*T* (°C)
0–6	6.5	3.0	0.2	52.1	8.8
6–11	6.5	2.1	0.1	56.2	5.2
11–18	6.3	2.3	0.1	59.5	3.5
18–25	6.4	2.0	0.1	140.6	2.4
25–32	6.3	1.2	0.0	176.5	0.9
32–38	6.1	2.8	0.1	n.d.	n.d.

n.d. Not determined

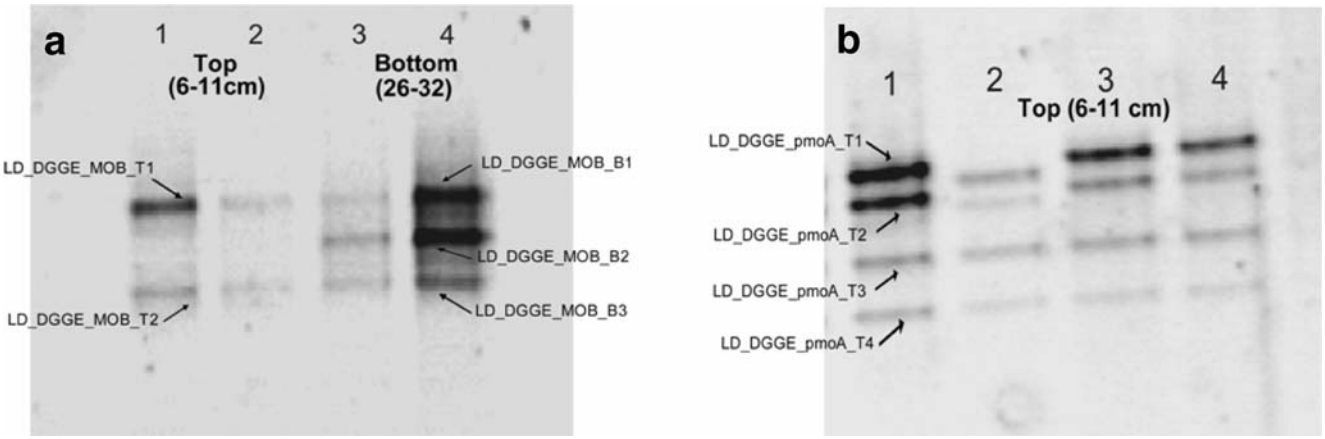

Figure 1 DGGE profiles of aerobic methanotrophic bacteria from active layer samples of Samoylov Island, Lena Delta. **a** Methanotroph-specific 16S rRNA gene fragments of near-surface (*Top*, *lines 1* and *2*) and near-permafrost table (*Bottom*, *lines 3* and *4*) samples and **b** *pmoA* gene fragments of near-surface samples (*lines 1–4*). *Arrow-marked bands* were excised and sequenced. *Lines* with bands that were not chosen for sequencing are shown to prove analytical reproducibility of the DGGE profiles

from *M. psychrophilus* and *M. tundripaludum* as their closest cultured relatives. The OTU of cluster III is directly affiliated to *M. psychrophilus* and *M. tundripaludum*. OTUs of cluster IV are directly affiliated to *M. fibrata* and *M. quisquiliarum* and are closely related to *Methylomicrobium buryatense*.

At the species level (≥98% sequence similarity), we detected a distinct overall diversity based on the Shannon index (2.22) that was similar near the surface and near the permafrost table (1.90). In addition, the overall evenness based on the Simpson index was almost equal in both depths (0.14 near the surface compared to 0.12 near the permafrost table). All diversity indices are summarized in Table 4. According to the estimated species richness (Chao1 and ACE estimators), overall coverage and coverage values of the near-surface and near-permafrost table libraries were high (76–100%). This is also reflected in the respective rarefaction curves (Fig. 3). Figure 3a includes an interpolation of the total rarefaction curve. It emphasizes that with 55 clones, we detected greater than 70% of the estimated richness of OTUs and that at least 100 more clones would be necessary to gain four OTUs more.

At the genus level (≥95% sequence similarity) [48], the overall diversity decreased significantly. Based on the Shannon index, it was only half of that at the species level. In addition, the estimated overall richness at the genus level was only 30% that of the species level.

Phylogenetic Analysis of the *pmoA* Gene

In addition to the diversity of MOB in active layer samples of two depths of a Siberian permafrost active layer soil based on the 16S rRNA gene, we investigated the distribution and phylogenetic relatedness of *pmoA* gene sequences in the same samples.

Table 3 Phylogenetic characterization of 16S rRNA gene sequences of aerobic methanotrophic bacteria of active layer samples from Samoylov Island, Lena Delta, obtained through DGGE

Sequence ID[a] (sequence length)	Next cultured relative according to NCBI	Accession number, GenBank	Similarity according to NCBI BLAST[b] (%)	Similarity according to NJ[c] (%)
LD_DGGE_MOB_T1 (826 bp)	*Methylobacter psychrophilus*	AF152597	98	99
LD_DGGE_MOB_T2 (829 bp)	*Methylosarcina fibrata*	AF177296	99	99
	Methylosarcina quisquiliarum	AF177297	98	98
LD_DGGE_MOB_B1 (855 bp)	*Methylobacter psychrophilus*	AF152597	97	97
LD_DGGE_MOB_B2 (846 bp)	*Methylobacter tundripaludum*	AJ414655	98	99
LD_DGGE_MOB_B3 (753 bp)	*Methylosarcina fibrata*	AF177296	99	99
	Methylosarcina quisquiliarum	AF177297	98	98

[a] According to Fig. 1, *T* top, *B* bottom
[b] Nucleotide–nucleotide query, BLASTN algorithm
[c] *NJ* Neighbor-joining algorithm (distance matrix) with Felsenstein correction [16]

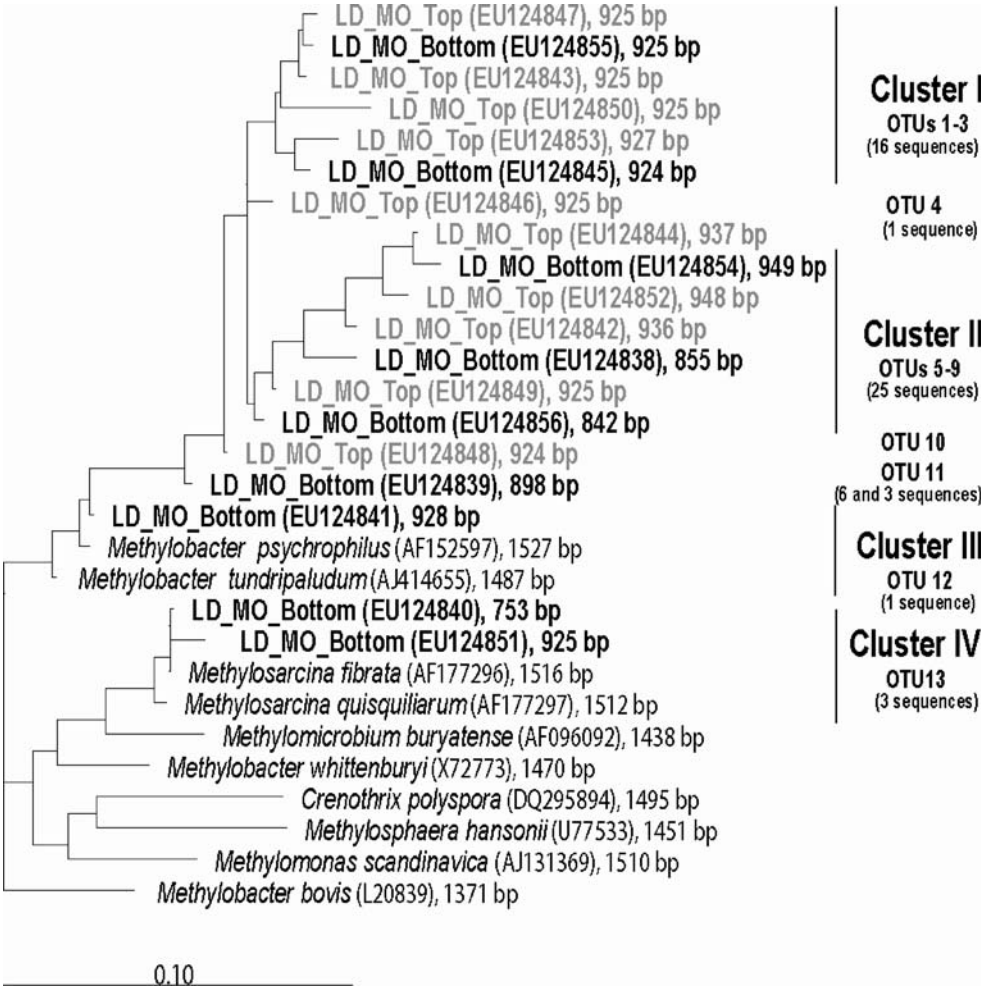

Figure 2 Phylogenetic tree showing the relation of 16S rRNA gene sequences of aerobic methanotrophic bacteria from active layer samples of Samoylov Island, Lena Delta, to most closely branching 16S rRNA gene sequences of known methanotrophic isolates and to sequences of selected methanotrophic isolates as references. The 16S rRNA gene sequences shown here were selected according to their affiliation to 13 different OTUs detected in this study. The tree represents a maximum likelihood tree based on the SILVA Ref dataset (www.arb-silva.de) and was calculated according to the PhyML algorithm [20] using a 50% filter for range of the PCR product specific for *Methylococcaceae*. We abstained from bootstrapping. *LD* Lena Delta, *Top* near-surface samples, *Bottom* near-permafrost table samples

Table 4 Diversity indices of 16S rRNA gene sequences of aerobic methanotrophic bacteria in active layer samples from Samoloy Island, Lena Delta

	DI[a]	Total[b]		Top		Bottom	
		Sequence similarity					
		98%	95%	98%	95%	98%	95%
	Shannon	2.22	1.01	1.90	0.81	1.90	1.12
	Min, max[c]	2.2, 2.5	1.0, 1.4	1.7, 2.1	0.6, 0.9	1.6, 2.2	0.7, 1.6
	Simpson	0.10	0.34	0.14	0.45	0.12	0.41
	Chao1	17	5	9	3	8	n.d.[d]
	Min, max[c]	14, 37	5, n.d.[d]	8, 22	3, n.d.[d]	8, 12	
	ACE	17	5	10	n.d.[d]	9	n.d.[d]
	Min, max[c]	15, 25	5, 13	9, 17		8, 18	
Coverage (%)	Chao1	76	100	100	100	100	n.d.[d]
	Min, max[c]	35, 92	n.d.[d], 100	41, 112.5	100, n.d.[d]	66, 100	
	ACE	76	100	90	n.d.[d]	88	n.d.[d]
	Min, max[c]	52, 86	38, 100	52, 100		44, 100	

[a] Diversity index, calculated with DOTUR [49], neighbor-joining algorithm (distance matrix) with Felsenstein correction [16], Diversity indices are based on 16S rRNA gene sequences from clone libraries only
[b] Sequences near the surface (Top) and near the permafrost table (Bottom)
[c] According to confidence interval of 95%
[d] Not determined

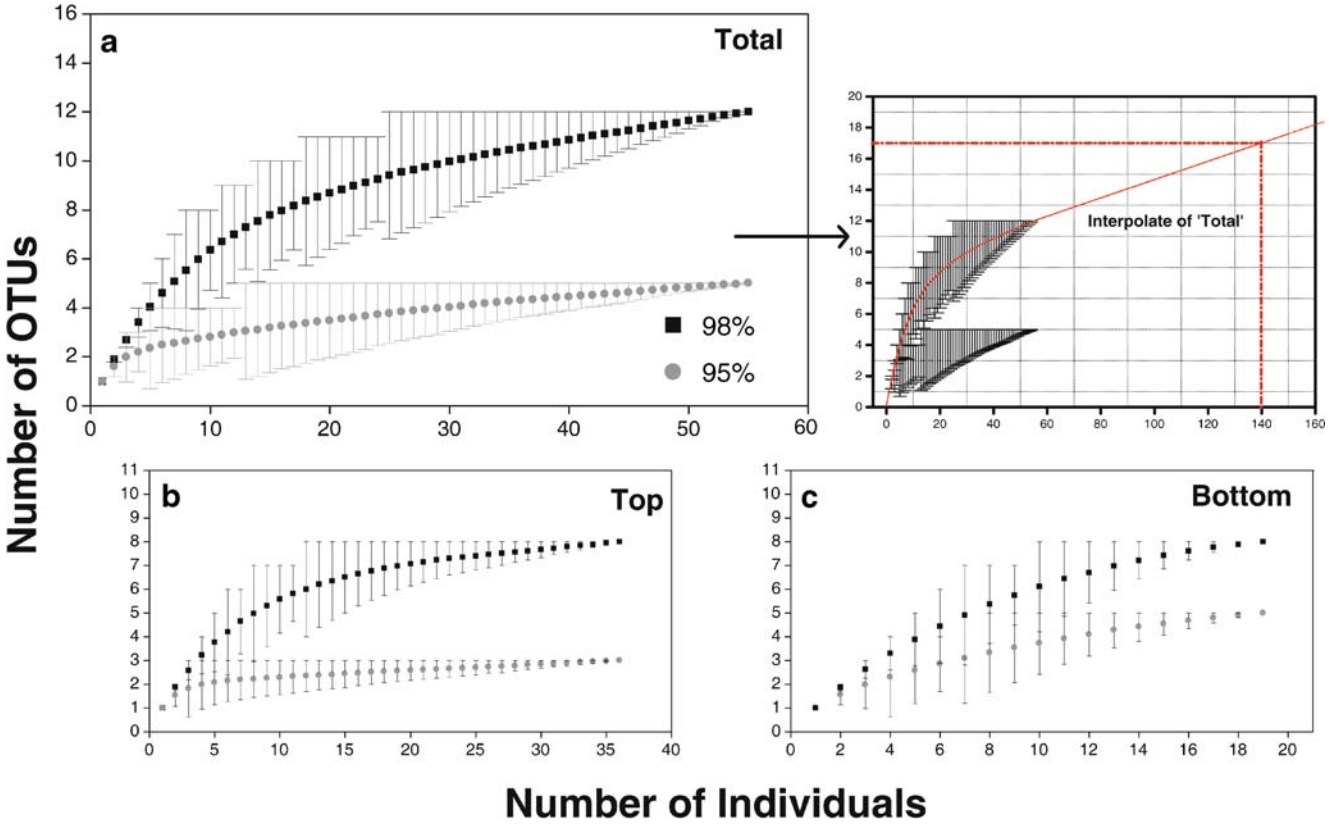

Figure 3 Rarefaction curves of 16S rRNA gene sequences of aerobic methanotrophic bacteria from active layer samples of Samoylov Island, Lena Delta. Graphs show a comparison between 98% and 95% sequence similarity of **a** near surface+near permafrost table samples, *arrow* marks interpolated number of clones necessary to obtain estimated species richness of 17 OTUs, **b** only near-surface and **c** only near-permafrost table samples. *Error bars*, confidence interval of 95%

DGGE profiles of the *pmoA* gene could only be obtained for the near-surface samples. We reproducibly detected four distinct bands (Fig. 1b) that were excised and sequenced. DGGE bands of the near-permafrost table samples could not be excised and sequenced successfully as a result of very low amounts of template. For this reason and in order to confirm the DGGE results of the near-surface samples, we constructed small clone libraries of the *pmoA* gene for the near-surface and the near-permafrost table samples. We obtained 18 clones that gave five additional *pmoA* gene sequences different from the previous ones.

The distribution and phylogeny of the *pmoA* gene sequences was consistent to the distribution and phylogeny of the 16S rRNA gene sequences. Again, we did not detect *pmoA* gene sequences belonging to the type II group of MOB, and the phylogenetic distribution of sequences was restricted (Fig. 4). The *pmoA* gene sequences obtained in this study grouped in four clusters (clusters I to IV in the tree). The sequence similarity of the representatives of clusters I and II to *M. psychrophilus* and *M. tundripaludum* was greater than 80%. The sequence similarity of representatives of cluster III to *M. buryatense* was greater than 80%. Representative sequences of cluster IV were less than 80% similar to any known isolate.

Discussion

Our results showed that members within the type I MOB were closely related to only two known genera, *Methylobacter* and *Methylosarcina*. This was consistently detected applying two different methods, DGGE and cloning. Given that altogether ten genera belong to the type I MOB [5, 25, 45, 54, 60], this points at a restricted diversity within the studied community of this group. In terms of relative abundance, representatives related to the *Methylobacter* genus were clearly dominant, whereas the number of representatives of the *Methylosarcina* genus was only marginal. In a related study on high-arctic sediments based on DGGE profiles, the MOB community was also found to be restricted to only two genera [57]. In that study, members of the genera *Methylobacter* and *Methylosinus* and consequently both type I and type II MOB were detected. Similar to our study members of the *Methylobacter* genus were recovered to be dominant.

A low diversity or a restriction to certain groups of MOB was reported also for nonarctic environments that exhibit extreme environmental conditions. The MOB community of peat bogs or acidic forest soils, for example, was restricted to members of type II MOB [39, 40, 43]. Besides, all known

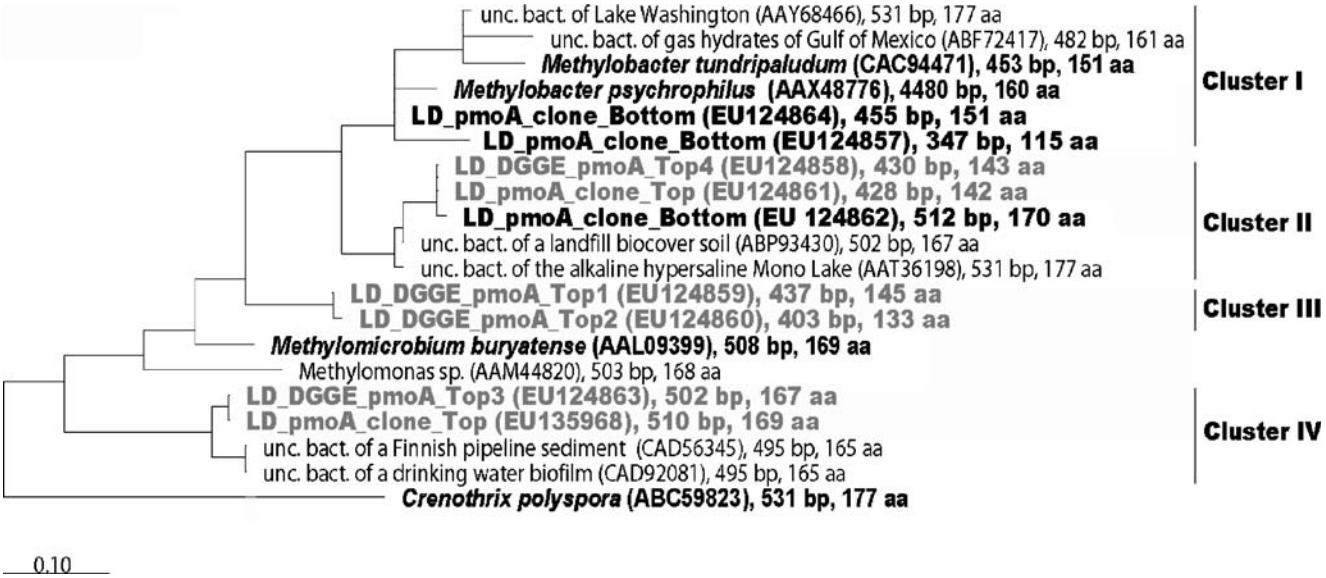

Figure 4 Phylogenetic tree showing the relation of representative amino acid sequences (translated *pmoA* gene sequences) from active layer samples of Samoylov Island, Lena Delta, to most closely branching *pmoA* gene sequences of cultured and uncultured aerobic methanotrophic bacteria and to *Crenothrix polyspora* (outgroup sequence). The tree represents a maximum likelihood tree calculated according to the PhyML algorithm [20] using a 30% filter (amino acid position 63–201). We abstained from bootstrapping. *LD* Lena Delta, *Top* near-surface samples, *Bottom* near-permafrost table samples, *DGGE_pmoA* sequences obtained through DGGE, *pmoA_clone* sequences through clone libraries

acidophilic isolates belong to the genera *Methylocapsa* and *Methylocella*, both type II MOB [9–12]. The MOB community capable of the consumption of atmospheric methane was studied in different forest and grassland soils and was found to exclude members of the type I group. It was suggested to consist primarily of type II MOB [35] and also of distinct, uncultivated clades distant from type I and type II MOB [23, 29, 33, 46]. In all studies discussed, the MOB community is exposed to environmental extremes such as freeze–thaw cycles, low pH values, or low substrate concentrations and was found to be restricted to certain taxonomic groups of MOB. In contrast to that, a wide range of MOB genera of both type I and type II MOB was reported for more moderate environments such as landfill soils [59], rice field soils [22, 24, 28], freshwater sediments [8, 42, 44], and subarctic tundra soils [31]. This points at a trend toward a restricted diversity or a selection for certain groups of MOB in more extreme environments, which is in accordance with the results obtained in the present study.

The absence of type II MOB in our study on the 16S rRNA as well as on the *pmoA* gene level confirms a previous study indicating a clear dominance of type I over type II MOB in active layer samples from Samoylov Island using phospholipid fatty acid (PLFA) analysis [55]. Our methodological approach is based on primer combinations that were designed to detect both 16S rRNA and *pmoA* gene sequences of known and unknown type I as well as type II MOB [3, 8, 59]. These primer combinations were used in several studies where type I and type II MOB were detected either based on the 16S rRNA gene [57], based on the *pmoA* gene [29, 42], or based on both genes [28, 44]. According to this, we would have detected type II MOB if their abundance was sufficiently high. However, type II MOB were below the detection limit in the studied permafrost active layer soil, while cell counts of type I MOB were in the range of 10^7 cells g^{-1} soil. The unsuccessful amplification of type II MOB could therefore be due to a PCR bias against this group of MOB as an artifact caused by a too low abundance of type II MOB in our samples. Previous studies showed that true psychrophilic MOB can be only found within the subgroup of type I MOB [4, 41], which is in accordance with in situ temperatures of permafrost environments. Considering this and the fact that type II MOB outcompete type I MOB only at either extremely low methane concentrations as discussed in the beginning or at distinctly heightened methane concentrations [38], it is evident that the environmental conditions of the Siberian permafrost lead to the dominance of type I MOB over type II species.

Based on 16S rRNA gene clone libraries, we found a distinct species-level diversity of the type I MOB within the active layer including two new clusters (clusters I and II) of MOB that were specific for the permafrost active layer soil studied here. More than 60% of all OTUs and more than 70% of all sequences detected belong to these clusters. Specific clusters for Siberian permafrost-affected soils based on the 16S rRNA gene could already be detected for other groups of microorganisms such as methanogenic archaea [19], which was interpreted as clusters formed by methanogens characterized by a specific adaptation to the

harsh permafrost conditions. The closest cultured relatives of members of the clusters detected here were *M. psychrophilus* and *M. tundripaludum*. *M. psychrophilus* was isolated from Siberian tundra and was characterized as a true psychrophile with an optimum growth temperature between 3.5°C and 10°C [41]. *M. tundripaludum* was isolated from an Arctic wetland soil on the island of Svalbard, Norway, and was characterized as a psychrotroph [58]. The sequence similarity of representatives of clusters I and II to *M. psychrophilus* and *M. tundripaludum* was less than 95%. Considering the 16S rRNA sequence similarity only, these clusters can be distinguished from known genera [48].

Our additional analysis of the phylogeny and distribution of the *pmoA* gene revealed a remarkable congruence with the phylogeny of the 16S rRNA gene sequences. The *pmoA* cluster I coincided with the two specific 16S rRNA gene permafrost clusters (clusters I and II), whereas *pmoA* cluster II confirmed cluster III in the 16S rRNA tree. The first group of sequences was directly affiliated to the species *M. psychrophilus* and *M. tundripaludum*. The second group of sequences was not directly affiliated to any known species, but the closest cultivated relatives were again *M. psychrophilus* and *M. tundripaludum*. Based on this, the majority of MOB detected in this study was found to be closely related to phsychrophilic and psychrotrophic MOB isolated from Arctic environments. In addition, there is indication of a vertical shift in MOB population. Based on the 16S rRNA gene sequences, clusters III and IV are exclusively bottom. With respect to the *pmoA* gene sequences, cluster I is exclusively bottom, too, whereas III and IV are top. Although it is difficult to deduce a straight phylogenetic relationship between the distinct clusters of environmental sequences detected, the similarity in the 16S rRNA and *pmoA* gene sequence clustering suggest that species of the same type I lineages were detected by both approaches. In addition, the comparison of the diversities obtained by analysis of both genes covered similar ranges.

Based on our data, we were able to compare the diversity of the active layer MOB near the surface and near the permafrost table. This comparison was necessary as we were aiming at investigating whether the gradients of temperature and methane within the active layer influence the diversity of the MOB community. Although, compared to the near surface, the active layer zone close to the permafrost table is characterized by a negative redox potential [17], by temperatures constantly below 2°C, and significantly lower cell counts of MOB [37], diversity and estimated richness values were found to be almost equal in both depths. Thus, the gradients within the studied active layer do not seem to influence the diversity of the MOB, although they do influence their abundance and, as discussed before, most likely their community structure. This indicates that, firstly, the gradients within the active layer are too weak to affect the diversity of the MOB community. Secondly, diversity and abundance must be regulated by different parameters. This is in accordance with results obtained from the phospholipid biomarker analysis. The PLFA distribution of near-surface and near-permafrost horizons of the active layer showed that the microbial communities of both horizons do not incorporate significantly more unsaturated fatty acids under cooler conditions (Mangelsdorf and Wagner, personal communication). This indicates a high level of adaptation to low temperatures of permafrost microbial communities in general.

Conclusions

With this study, we present first data on the diversity of the MOB community in an Arctic permafrost affected environment from northeast Siberia at a high phylogenetic resolution. The spatial and temporal "snapshot" of methanotroph diversity presented here revealed a community of MOB that is specialized to the extreme environment it is exposed to. We showed that, in contrast to a distinct species-level diversity, the diversity of the MOB community at a higher taxonomic level is restricted to members closely related to only two known genera. The presented data support a trend reported in other studies toward a restricted diversity of MOB in more extreme environments. In the context of the climatic changes presently affecting the Siberian Arctic and its polygonal tundra landscapes, a restricted diversity could negatively affect the community of MOB and their function as a primary sink for methane in these regions.

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