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Detection of methanotrophs with highly divergent *pmoA* genes from Arctic soils

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Abstract

Tundra soil samples from the Canadian Arctic community, Kuujjuaq, were analyzed for the presence of the soluble (sMMO) and particulate (pMMO) methane monooxygenase genes. Total genomic DNA extracted from these soils was used as template for PCR using sMMO- and pMMO-specific primers, *mmo*X1–*mmo*X2 and A189–A682, respectively. pMMO and sMMO genes were detected in the Kuujjuaq soil samples. Isolation of sMMO-possessing methanotrophic microorganisms from the three soils, as determined by the colony naphthalene oxidation assay, was carried out using direct plating (5°C) and methane enrichment studies (5°C and 25°C). Direct plating did not yield sMMO-possessing methanotrophic bacteria, whereas methane enrichments yielded isolates possessing and expressing sMMO activity. Analysis of derived amino acid sequences of *pmoA* genes and partial 16S rRNA genes obtained by PCR, using DNA isolated directly from this environment and from isolates, revealed the presence of highly divergent PmoA/AmoA sequences and 16S rRNA sequences that cluster closely with but are distinct from the genes from the genera *Methylosinus* and *Methylocystis*. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Methanotroph; Methane monooxygenase gene; 16S rRNA

1. Introduction

Methane-oxidizing bacteria (methanotrophs (MOB)) play an essential role in the global carbon cycle. They are responsible for most of the biological methane oxidation, thus preventing methane accumulation in the atmosphere. Methanotrophs obtain all of their carbon and energy from methane. The oxidation of methane to methanol, the first step in the process, is catalyzed by methane monooxygenase (MMO), of which two types have been identified: a membrane-bound or 'particulate' MMO (pMMO) and a cytoplasmic or 'soluble' MMO (sMMO). Methanotrophs are classified into two distinct taxonomic groups. Type I methanotrophs belong to the γ-subdivision of the Proteobacteria, whereas type II methanotrophs belong to the α-subdivision of the Proteobacteria [7]. All known methanotrophs (type I and type II) possess the pMMO. In contrast, the sMMO is restricted to type II methanotrophs with the exception of certain representatives of two type I genera (*Methylococcus* and *Methylomonas*) [19]. Expression of the sMMO is negatively regulated by high copper/biomass ratios in the growth medium [17]. The sMMOs from four strains belonging to three genera (*Methylocystis*, *Methylosinus*, and *Methylococcus*) have been thoroughly studied at the biochemical and genetic levels [2,5,6,17]. The sMMOs from these strains possess broad substrate specificity and can oxidize a wide variety of chlorinated and non-chlorinated aliphatic, alicyclic and aromatic compounds. The gene sequence of the sMMO cluster from these strains appears to be highly conserved. In contrast to the sMMO, the pMMO and more specifically the *pmoA* gene, which encodes a 26-kDa subunit believed to harbor the active site of the enzyme, possesses sufficient sequence divergence to serve as a diagnostic gene/enzyme for different methanotrophs [16].

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Molecular ecology techniques involving 16S ribosomal technology and specific amplification of 'functional genes' such as those encoding the enzymes pMMO and methanol dehydrogenase have been utilized to determine methanotroph diversity in various environments [3,8,10,14–16,18]. Although certain environments have been extensively studied for the presence and diversity of methanotrophic bacteria (e.g. freshwater and marine sediments, peat bog, waste-treating anaerobic bioreactors, soils and groundwater) Arctic environments remain essentially uncharacterized. Furthermore, most of the known physiological and molecular information originates from mesophilic and, to a lesser extent, thermophilic methanotrophic isolates. Since very little is known of the abundance, diversity and physiology of psychrotrophic methanotrophs, the aim was to investigate soils from the Canadian Arctic in order to expand our knowledge of the microorganisms inhabiting this environment. As part of a study assessing bioremediation of hydrocarbon-contaminated soils [21], type II methanotrophs, and more specifically sMMO-possessing methanotrophs, were targeted in this study.

2. Materials and methods

2.1. DNA extraction from environmental samples and enrichment cultures

Surface soils (vertical cores ranging from 15 to 30 cm in depth) were collected, mid summer (July), from petroleum hydrocarbon-contaminated sites K1 and K2 (containing 600 and 1400 ppm of total petroleum hydrocarbons, respectively) and from a pristine site (K3) at Kuujjuaq in Northern Quebec, Canada [21]. The soils were placed over ice and transported to Montreal for analysis. For direct cultivation (non-enrichment) of psychrotrophic sMMO-possessing methanotrophs, serial dilutions of soil samples were plated on copper-free LN-NMS plates [18]. Plates were placed in modified 'anaerobic jars' flushed with a 25–75% methane–air mixture and incubated at 5°C for 8–10 weeks. For enrichment of sMMO-possessing methanotrophs, duplicate soil samples (5 g) were added to 100-ml serum bottles containing 20 ml copper-free LN-NMS. The flasks were supplied with a 25–75% methane–air mixture, flushed weekly and incubated at 25°C for 2–10 weeks with shaking at 250 rpm. Duplicate samples were incubated at 5°C.

Direct DNA extraction from soils was performed using the method of Flemming et al. [4]. Soils (10 g) were placed in 30-ml Teflon centrifuge tubes and 4.5 ml H_2O was added. To these soil suspensions, 500 μl of 250 mM Tris–HCl (pH 8.0) containing 50 mg lysozyme was added. Mixtures were incubated for 30 min at 30°C with agitation (400 rpm) followed by 30 min incubation at 37°C. Proteinase K was added to a final concentration of 100 μg ml^{-1}. Incubation at 37°C for 30 min was followed by the addition of 500 μl filter-sterilized SDS (20%). Samples were then incubated for 30 min at 85°C, followed by centrifugation to remove soil particles (14 000 × *g*, 10 min). Supernatants were treated with 1/2 volume of 7.5 M ammonium acetate to precipitate proteins and humic acids. Samples were placed on ice for 5 min and centrifuged (12 000 × *g*, 3 min). Supernatants were then transferred to clean tubes and one volume of cold isopropanol was added followed by DNA precipitation (16 h at −20°C). Samples were centrifuged (12 000 × *g*, 15 min), supernatants were discarded and pellets washed with 70% (v/v) ethanol followed by centrifugation (12 000 × *g*, 15 min). Supernatants were discarded and pellets dried under vacuum. DNA in the pellets was resuspended in 350 μl TE buffer and purified further using MicroSpin columns (Pharmacia). This method yielded high quality DNA as determined by the ability to amplify 16S rRNA genes using universal eubacterial primers.

Crude DNA was obtained from enrichment cultures (single colonies) by boiling cell suspensions in water for 10 min, and removing cellular debris by centrifugation (20 800 × *g* for 2 min).

2.2. PCR amplification, cloning of amplified products and sequencing of PCR fragments

Functional (*mmoX* and *pmoA*) and 16S rRNA genes were amplified from all DNA samples as previously described [16,18]. Sequences of primers used for amplification of functional genes (*mmoX* and *pmoA*) and phylogenetic (group-specific 16S rRNA) have been previously described [3,10,16,18]. Isolate DNA was also tested for amplification by PCR using 16S rRNA specific primers designed to amplify genes from ammonia oxidizers belonging to the β-sub-group of the Proteobacteria [13]. Reaction products were verified for size and purity (1.2% w/v agarose gels), cut and extracted from the gel with the Qiaex II gel extraction kit (Qiagen). Purified DNA was cloned in a pCR2.1-TOPO vector with the TOPO TA cloning kit (Invitrogen) in the competent *Escherichia coli* strain TOP 10. Plasmid DNA was isolated using the QIA Spin mini-prep kit (Qiagen). PCR sequencing was carried out with the ABI PRISM dRhodamine Terminator cycle sequencing kit (Applied Biosystems). Nucleotide and inferred polypeptide sequences were aligned with sequences obtained from GenBank using the ARB program for sequence alignment (http://www.mikro.biologie.tu-muenchen.de), and the phylogenetic position determined using programs of the PHYLIP package. Sequence comparison, multiple alignment and phylogeny were also performed using the Mac Vector program (Oxford Molecular).

Table 1
Similarity matrix comparing derived PmoA/AmoA amino acid sequences of methanotrophs and nitrifiers generated by PCR amplification

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1.	Tundra Soil K1 (2)	100	85	74	77	63	49	82	89	58	83	53	53	42	52	41	40
2.	Tundra Soil K2 (2)		100	85	71	74	54	96	91	67	94	56	56	42	52	41	39
3.	Tundra Soil K3 (2)			100	65	85	47	86	78	54	84	49	49	37	45	36	34
4.	Isolate 1				100	44	59	71	74	49	70	44	44	37	46	37	34
5.	Isolate 8					100	41	75	66	48	73	42	40	31	40	30	28
6.	Isolate 10						100	54	52	38	55	87	90	50	46	36	47
7.	Isolate 14							100	87	64	93	56	55	41	51	40	38
8.	Isolate 16								100	62	87	54	53	40	50	39	37
9.	Isolate 17									100	63	40	39	30	36	27	28
10.	Isolate 21										100	56	56	42	53	41	40
11.	*M. trichosporium*											100	93	54	49	39	49
12	*Methylocystis* sp. M												100	52	48	38	48
13	*M. capsulatus*													100	60	61	77
14	*Nitrosospira* sp.														100	75	55
15	*N. europaea*															100	57
16	*Nitrosococcus oceanus*																100

3. Results and discussion

3.1. Physico-chemical characteristics of soil samples and isolation of methanotrophic bacteria

The goal of this study was two-fold: (i) to isolate sMMO-possessing methanotrophs from Arctic Tundra soil samples; (ii) to study the phylogenetic diversity of methanotrophs and MMO genes in selected Arctic soils.

The biological and physico-chemical characteristics of the soil samples utilized in this study have been described in detail [21]. The soils are neutral to slightly alkaline (pH ranging from 7.2 to 7.7). Total heterotrophic bacterial counts range from 5.00×10^6 to 5.99×10^7 [21]. Soil K3 (pristine) is peat-like in nature, in contrast to the predominantly gravel-like K1 and K2 soils. The carbon content of soils K1 and K2 is 0.62% and 0.69%, respectively. The pristine peat-like soil has a significantly higher carbon content of 9.43%.

Direct cultivation and a methane enrichment study were undertaken. In order to favor the chances of isolating sMMO-possessing methanotrophs, a sMMO bias was imposed on the isolation protocols by utilizing a growth medium devoid of copper. Even after several weeks of incubation, we were unable to isolate psychrotrophic sMMO-possessing methanotrophs, either by direct plating or in enrichment cultures (incubation at 5°C), from any of the soils tested. Replicate methane enrichment samples, incubated at 25°C, did however yield type II (sMMO-possessing) methanotrophs. Two criteria were utilized for the selection of methanotrophs: (i) the ability to oxidize naphthalene, an important physiological trait which differentiates methanotrophs which express the sMMO from those that possess only the pMMO [1]; (ii) PCR amplification of template DNA with *pmoA* and *mmoX* gene-specific primers. Methanotrophs obtained from methane enrichments at 25°C did not grow on or consume methane at 5°C. These results are not entirely surprising. The inability to isolate psychrotrophic type II methanotrophs has also been observed by others [12,20]. Since a high methane concentration was used in the isolation protocols, methane oxidizers that oxidized atmospheric concentrations of methane (2 ppm) may have been overlooked.

3.2. Characterization of pmoA, mmoX and 16S rRNA genes from total community soil DNA

The *pmoA*-specific primers (primers A189 and A682) used to amplify partial *pmoA* gene products from Kuujjuaq soil DNA are degenerate primers in that they also amplify the *amoA* gene (encoding the 27-kDa ammonia monooxygenase subunit) of nitrifiers belonging to α- and β-Proteobacteria [16]. Phylogenetic analysis of the derived amino acid sequences of *pmoA* genes obtained from Kuujjuaq soil DNA reveal novel PmoA/AmoA sequences with low similarities to known PmoA/AmoA from methanotrophs and nitrifiers (Table 1). A more detailed analysis of the derived amino acid sequences (Fig. 1) revealed that although these sequences cluster closer to nitrifiers (β-subdivision), they do form a distinct branch. Recently Henckel et al. [8] described derived PmoA/AmoA sequences from forest soil (atmospheric methane oxidizers) which were most closely related to *Nitrosospira* but sufficiently distinct to branch separately. However these sequences grouped closer to a sequence from an uncultured methanotroph (RA21), previously detected in an atmospheric methane-oxidizing forest soil [9], and not with the sequences detected in the Kuujjuaq soils (Fig. 1). Many of the conserved amino acid residues of known PmoA/AmoA sequences are present in the Kuujjuaq soil sample sequences (Fig. 2), strongly suggesting that these sequences are indeed PmoA/AmoA. Universally conserved MOB residues present in K1, K2 and K3 are 80% (41 of 51), 78% (38 of 49) and 76% (37 of 49), respectively. The amino acid

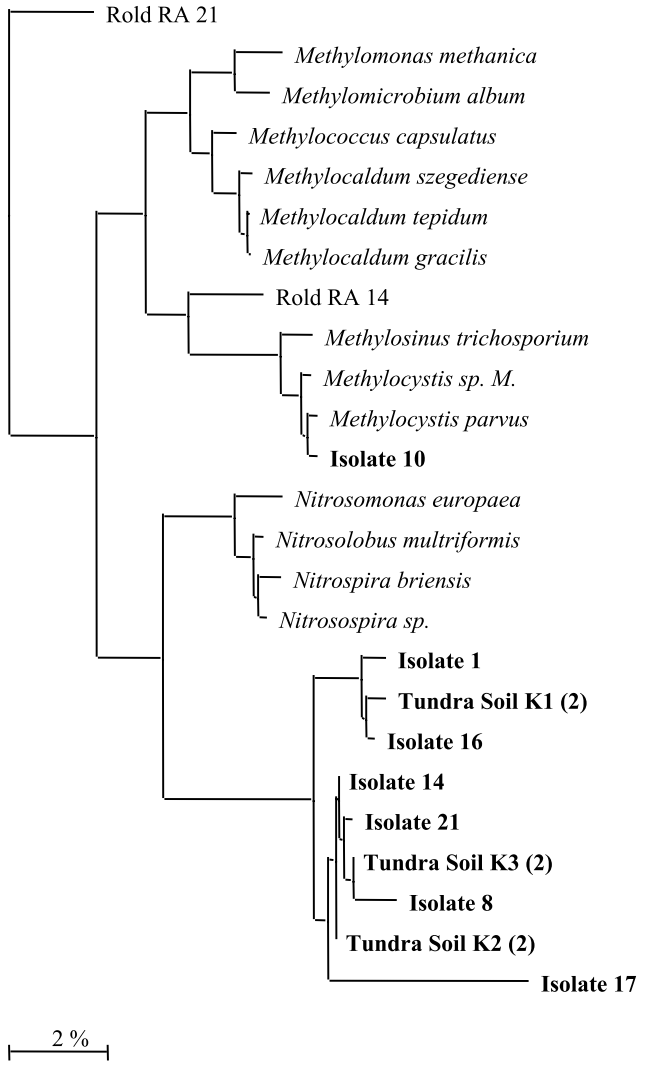

Fig. 1. Phylogenetic analysis of the derived amino acid sequences (170 amino acids) of PmoA/AmoA genes from methanotrophs, nitrifiers, tundra and isolate DNA. The dendrograms show the results from analysis using neighbor-joining and Poisson correction. The bar represents 2% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two species.

sequences contain 31 out of 60 residues inferred to the AMO and only four out of 27 putative to the MMO. Therefore, this clearly indicates a bias towards the AMO signature. A new reverse primer mb661 has been described which, when used in conjunction with the degenerate forward primer A189, specifically amplifies *pmoA* sequences [3]. These primers failed to amplify template DNA from all of our Kuujjuaq soil samples suggesting a lack of target in these soils.

Primers previously shown to amplify partial (500 bp) *mmoX* sequences from extant sMMO-possessing methanotrophs [18] were used in this study. Sequence analysis of the MmoX fragments obtained from total community DNA revealed a high degree of identity (95–97%) with the MmoX of *Methylosinus trichosporium*. Unlike the *pmoA* gene, *mmoX*, and presumably the sMMO cluster from all known cultures and reported environmental sequences, appears to be highly conserved.

Characterization of 16S rRNA genes from total community soil DNA was done using *Methylosinus*/*Methylocystis* genus-specific primers, Ms1020 and type 2b [3,11] in conjunction with the eubacterial-specific primer f27 [11]. However, PCR amplification was not obtained with any of the Kuujjuaq soil DNA samples when the *Methylocystis*/*Methylosinus*-specific primer Ms1020 was used. The control amplification using both universal eubacterial primers (f27–r1492) worked well, demonstrating that the DNA was PCR-amplifiable. One reason for the lack of amplification using primer Ms1020 may be that insufficient target for this primer is present in the soil DNA. However, amplification was obtained with the alternate primer type 2b, which has been shown by others [3] to amplify a broader spectrum of *Methylocystis*/*Methylosinus* 16S rRNA sequences (Fig. 3). 16S rRNA sequences obtained from Kuujjuaq soils clustered with type II methanotrophs. Although they closely resemble those from *Methylocystis*/*Methylosinus* species, they branch separately, suggesting that novel methanotrophs may be present. Unexpectedly, primer type 2b also amplified sequences which clustered closely with the genus *Methylobacterium*. This indicates that the specificity of primer type 2b may be broader than previously expected. This observation may limit the value of primer type 2b as a *Methylocystis*/*Methylosinus*-specific probe. The combination of novel 16S rRNA sequences and the atypical *pmoA*/*amoA* sequences obtained from the total community soil DNA suggests that the methanotrophic population in Kuujjuaq soils may contain novel MOB which are different to previously described isolates.

3.3. Molecular characterization of methanotrophs isolated in this study

Phylogenetic analysis of the derived amino acid sequences of *pmoA* genes obtained by PCR from isolates arising from methane enrichments revealed sequences with high identity to the highly divergent sequences obtained from the DNA extracted directly from the environment (Figs. 1 and 2 and Table 1). Other than for one isolate, high percentages were noted for the degree of universally conserved MOB residues, 74–98%. Isolate 10 also demonstrates a very high MMO signature. Twenty-five of the 26 MMO residues are present in the derived amino acid sequence for this isolate DNA. The remaining isolates are highly divergent from extant methanotroph signatures. They possess only 10–15% MMO residues while displaying 48–52% of the AMO signature residues. Since degenerate primers A189–A682, used to obtain these sequences, also amplify the *amoA* gene of nitrifiers, PCR using 16S rRNA specific primers designed to amplify genes from ammonia oxidizers belonging to the β-sub-group of the Proteobacteria [13] was performed on isolate DNA. No amplifica-

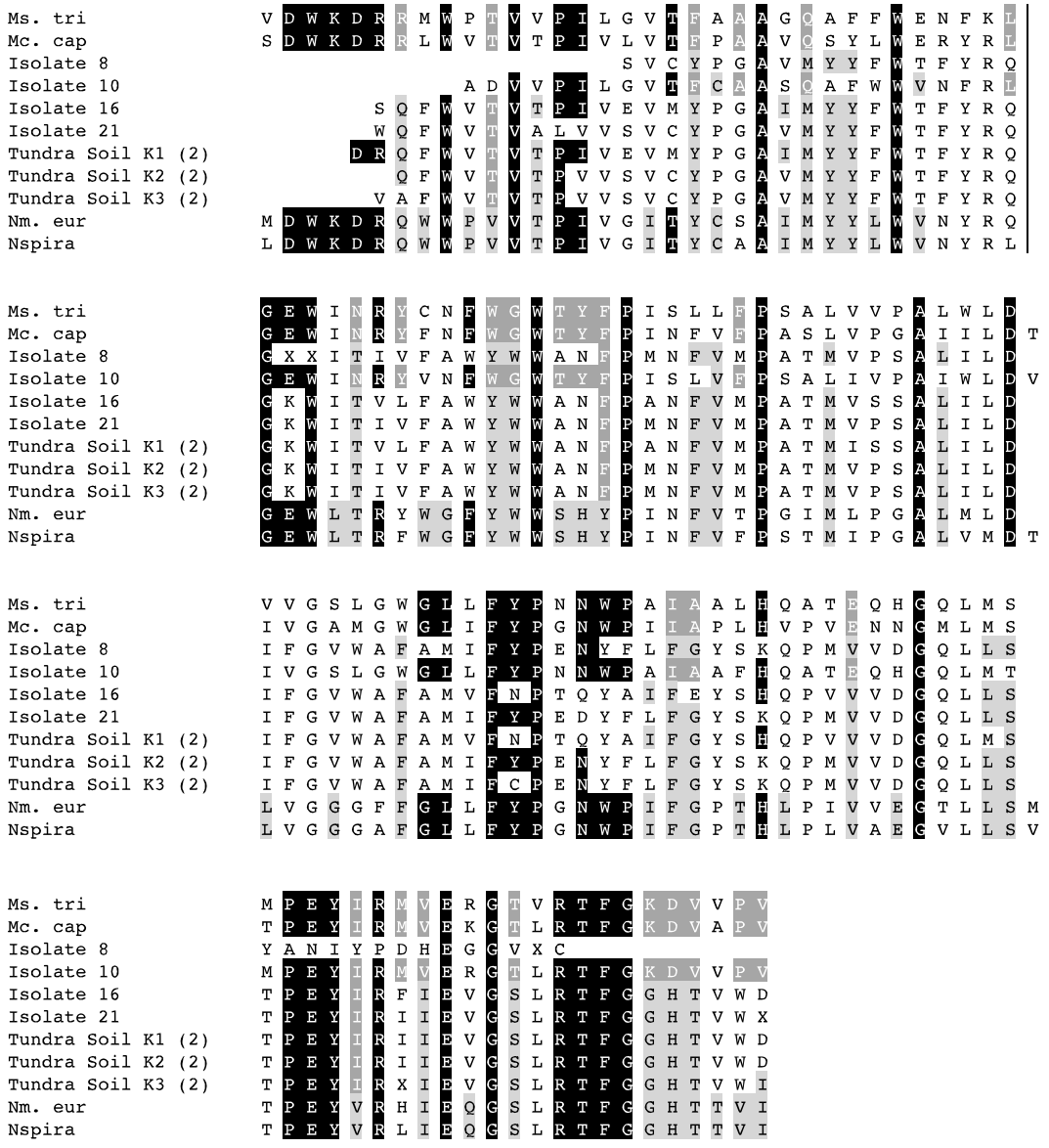

Fig. 2. Alignment of predicted peptide sequences of PmoA and AmoA from representative methanotrophic and nitrifying bacteria with Tundra and isolate DNA sequences was done as described by Holmes et al. [10]. Residues which are universally conserved in the extant MOB are highlighted with black. Putative MMO signatures are highlighted with white letters on a dark gray background and AMO signatures are highlighted with a light gray background. The sequences shown (and their accession numbers) are as follows: Ms. tri, *M. trichosporium* (MTU 31650); Mc. cap, *Methylococcus capsulatus* (L 40804); Nm. eur, *Nitrosomonas europaea* (L 08050); Nspira, *Nitrosospira* sp. (X 90821).

tion was obtained with the isolate DNA. Therefore, possible nitrifiers co-existing with the methanotrophs in culture were not detected. The fact that these methanotrophs oxidize methane and naphthalene, and possess the *mmoX* gene confirms that the 'novel' sequences, obtained from the total community and isolate DNA, are indeed *pmoA*. Methanotroph (isolate 10) with 'typical' type II *pmoA* sequence (i.e. sequence clustering closely with *Methylosinus*/*Methylocystis*) was also obtained (Table 1 and Figs. 1 and 2). However, this type II *pmoA* sequence was not detected in the total community DNA, perhaps indicating a low level of *Methylosinus*/*Methylocystis* representatives in the Kuujjuaq soil samples tested. Only as a result of enrichment was this sequence identified. Another effect of methane enrichment of soil samples is that the reverse *pmoA*-specific primer (mb661) which failed to amplify total community DNA from all Kuujjuaq soil samples did amplify DNA from half of the methanotrophs enriched from these same soils (results not shown). Unlike the degenerate primer which detected atypical PmoA/AmoA and typical type II PmoA sequences, only typical type II PmoA sequences were obtained with the mb661 *pmoA*-specific primer. These methanotrophs may be present in low numbers in the Kuujjuaq soils tested (i.e. sufficiently low as to not be detected by PCR using the mb661 primer set). This confirms the observation made by Costello et al. [3] that primer mb661, when used in conjunction with A189, only amplifies *pmoA* sequences.

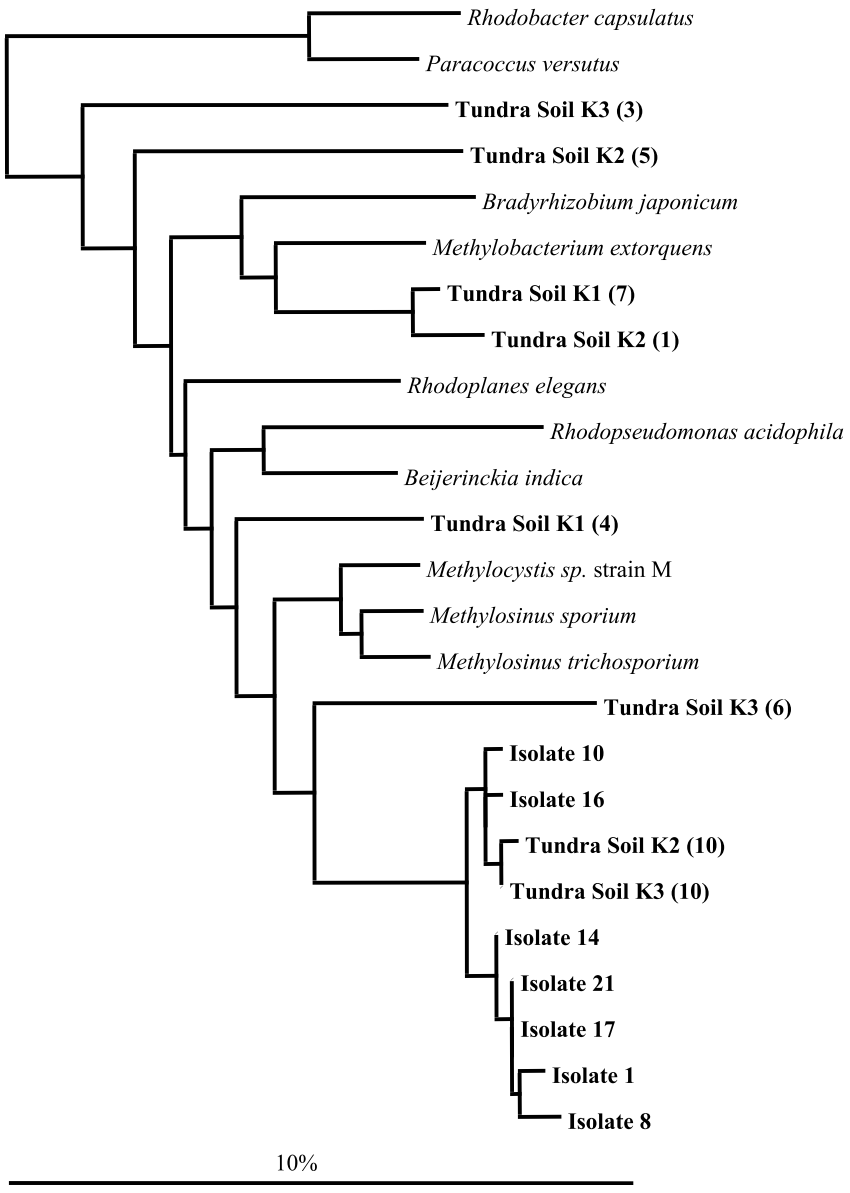

Fig. 3. Phylogenetic analysis of the 16S rRNA sequences (500 bp) from methanotrophs, methylotrophs and other representative bacteria belonging to α- and γ-Proteobacteria. Tundra and isolate sequences were amplified using primer type 2b. The dendrogram shows the result from analysis using DNADIST and neighbor-joining, and the bar represents 10% sequence divergence, as determined by measuring the lengths of the horizontal lines connecting any two species.

The 16S rRNA genes from isolated methanotrophs were analyzed using *Methylosinus*/*Methylocystis* genus-specific primers Ms1020 and type 2b. While primer Ms1020 failed to amplify 16S rRNA genes from Kuujjuaq soil DNA, both primers Ms1020 and type 2b amplified DNA from methanotrophs isolated from these soils. This confirms the observation that the lack of amplification when using primer Ms1020 is due to insufficient target present for this oligonucleotide since amplification was achieved with DNA from isolates obtained after enrichment culture with the same soil (results not shown). All methanotrophs isolated possessed type II 16S rRNA sequences clustering tightly with the 16S rRNA genes from *Methylosinus* and *Methylocystis*, but these 16S rRNA sequences did form a separate branch of the tree (Fig. 3). The seven isolates, represented on this 16S rRNA phylogenetic tree, originate from the three soils. Isolates 1 and 8 are from Tundra Soil K1. Isolates 10 and 14 are from Tundra Soil K2. Lastly, isolates 16, 17 and 21 originate from Tundra Soil K3. The grouping on the tree, for these isolates, does not reflect differences attributed to the origin of the soil. Therefore, the level of hydrocarbon contamination does not appear to affect the community structure of methanotrophs in this Arctic environment.

This study clearly demonstrates the importance of combining both the use of direct molecular microbial ecology

and the indirect enrichment/isolation strategies to enhance the potential of identifying methanotrophic populations present in the environment. Sole application of molecular biological tools would not have given a true representation of the methanotrophs present. Methanotrophs being less abundant in certain environments, as noted in the total community Arctic soil DNA, and considering that not all group-specific PCR primers are indeed specific, we would have missed some of these representatives had the study not combined both approaches. Given that enrichment yielded cultures with the peculiarity of closely resembling type II methanotrophs and possessing PmoA/AmoA sequences with high divergence to known cultures, these will be further studied to distinguish how the differences seen on a molecular level are reflected on the characterization of these microorganisms.

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