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Identification of the Functionally Active Methanotroph Population in a Peat Soil Microcosm by Stable-Isotope Probing

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The active population of low-affinity methanotrophs in a peat soil microcosm was characterized by stable-isotope probing. "Heavy" ^{13}C-labeled DNA, produced after microbial growth on $^{13}CH_4$, was separated from naturally abundant ^{12}C-DNA by cesium chloride density gradient centrifugation and used as a template for the PCR. Amplification products of 16S rRNA genes and *pmoA*, *mxaF*, and *mmoX*, which encode key enzymes in the CH_4 oxidation pathway, were analyzed. Sequences related to extant type I and type II methanotrophs were identified, indicating that these methanotrophs were active in peat exposed to 8% (vol/vol) CH_4. The ^{13}C-DNA libraries also contained clones that were related to β-subclass *Proteobacteria*, suggesting that novel groups of bacteria may also be involved in CH_4 cycling in this soil.

It is estimated that 400 to 640 Tg of methane (CH_4) is produced annually in anoxic environments (9), much of which is oxidized at the anaerobic-aerobic interface, thereby mitigating the global emissions of this greenhouse gas into the atmosphere. There are two distinct populations of organisms responsible for aerobic CH_4 oxidation. One population exhibits high-affinity CH_4 oxidation kinetics and acts as a sink for atmospheric concentrations of CH_4 in many soils (2, 8, 10, 15, 17, 20, 24). The second population (methanotrophs) can grow on CH_4 as a sole source of carbon and exhibits low-affinity CH_4 oxidation kinetics (2, 17), and extant strains can be divided into type I (γ-*Proteobacteria*) or type II (α-*Proteobacteria*) methanotrophs (4, 5, 7, 40).

Methane monooxygenase (MMO) and methanol dehydrogenase (MDH) are key enzymes involved in CH_4 oxidation. There are two forms of MMO, both of which oxidize CH_4 to methanol. A membrane-bound, particulate enzyme (pMMO) (32) has been reported in nearly all methanotrophs (12, 32). A soluble, cytoplasmic enzyme (sMMO) is only found in certain methanotrophs (32). The pMMO genes exhibit high similarity to genes encoding ammonia monooxygenase (23) from ammonia-oxidizing nitrifying bacteria (nitrifiers). Nitrifiers also oxidize CH_4 but probably do not use it as a carbon source (1). MDH oxidizes methanol to formaldehyde, and it is present in all known methylotrophic bacteria within the *Proteobacteria* (30). Genes encoding MMO and MDH (functional genes) and 16S rRNA genes have been used to assess the diversity of methanotrophs in the environment (11, 14, 18, 24, 31, 33, 44). Phylogenetic analysis of 16S rRNA, sMMO, pMMO, and MDH gene sequences obtained from these environments has suggested that a greater diversity of methanotrophs exists than we have seen in culture, a fact demonstrated by the continued isolation of novel methanotroph taxa (12, 25, 45).

A major goal of microbial ecology is to identify the microorganisms that are actively involved in specific processes in the environment. This has recently been addressed by using the natural abundance of stable isotopes in biomarkers (22, 34) and techniques including fluorescent in situ hybridization (FISH) coupled with microautoradiography (27, 35), analysis of phospholipid fatty acids by gas chromatography-isotope ratio mass spectrometry (3, 36), FISH coupled with mass spectrometry, and stable-isotope probing (SIP) (37, 41), which use substrates enriched with stable isotopes or radioisotopes. SIP exploits the fact that DNA of an organism growing on a ^{13}C-enriched carbon source becomes ^{13}C labeled ("heavier"), enabling it to be resolved from the total community DNA by density gradient centrifugation (37). The ability to isolate DNA from microorganisms involved in the metabolism of $^{13}CH_4$ allowed us to characterize the microbial population actively involved in low-affinity CH_4 oxidation in a peat soil microcosm without any prior knowledge of the organisms involved or the need to isolate them into culture.

Sample site and microcosms. The study site was drained fenland peat soil (49 to 51% carbon; pH 6.8) from Suffolk, United Kingdom [42]). The peat sample (0 to 5 cm in depth) was collected in May 1999 from an area not cultivated for 4 years. The in situ soil water content was 65% water-holding capacity, which was optimal for low-affinity (5% [vol/vol]) CH_4 oxidation (S. A. Morris, unpublished data).

Two microcosms were established for the SIP experiment. Each consisted of freshly collected peat soil (10 g at 65% water-holding capacity) in a 125-ml crimp-top serum vial, sealed with a butyl rubber stopper, and injected with 0.4 mmol (10 ml) of CH_4. One microcosm was exposed to $^{13}CH_4$ (99% pure, 99% ^{13}C atom enriched; Linde Gas), and the second microcosm was exposed to an identical amount of $^{12}CH_4$ (98% pure; Linde Gas). The $^{13}CH_4$ microcosm was used for extraction of ^{13}C-labeled DNA and characterization of the microbial population actively involved in low-affinity CH_4 oxidation.

Microcosms were incubated in the dark at 25°C, and headspace CH_4 concentrations (200-μl samples) were determined every 2 to 5 days by gas chromatography. After >90% of the CH_4 was consumed (7 to 12 days), the vials were flushed with

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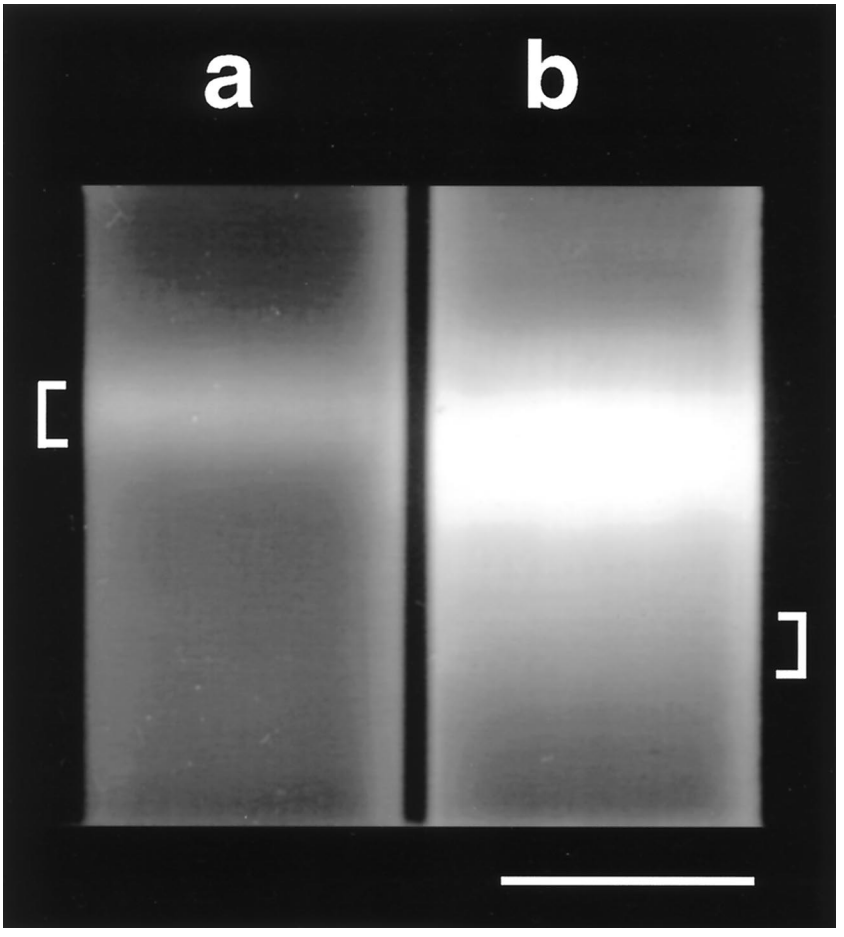


FIG. 1. Equilibrium centrifugation of DNA extracted from soil that oxidized $^{12}CH_4$ (a) or $^{13}CH_4$ (b). CsCl-ethidium bromide (100 μl at 10 mg ml^{-1}) density gradients were centrifuged at 265,000 × *g* for 16 h, and DNA was visualized with long-wavelength (365-nm) UV light. The positions of the ^{12}C-DNA (indicated by "[") and ^{13}C-DNA (indicated by "]") fractions collected after primary ultracentrifugation are indicated. Bar, 1 cm.

air to remove $^{13}CO_2$ and ensure that the microcosms remained aerobic. A further 0.4 mmol of $^{13}CH_4$ or $^{12}CH_4$ was added, and the vials were incubated until 1.6 mmol of CH_4 (four injections of 10 ml of CH_4) had been consumed by each microcosm (40 days).

DNA extraction. DNA was extracted from 10 g of soil exposed to $^{13}CH_4$ and 3 g of soil exposed to $^{12}CH_4$ by a bead-beating method (46) that was scaled up to process 3-g soil samples in 12-ml glass bead beater tubes (Braun). Soil samples were shaken for 5 min in a CO_2 cooled bead beater (Braun). Extracts from the $^{13}CH_4$ microcosm were pooled, and large particles were removed by centrifugation for 5 min at 120 × *g*. Proteins in the supernatant were precipitated with potassium acetate (7.5 M; one-sixth of the sample volume) and removed by centrifugation for 5 min at 15,000 × *g*. To the supernatant, an equal volume of binding matrix (Bio 101) diluted 1:5 with 6 M guanidine isothiocyanate was added. The tube was inverted regularly for 5 min and centrifuged for 5 min at 15,000 × *g*, and then the supernatant was discarded. The binding matrix was washed twice by resuspension in an equal volume of wash buffer (70% ethanol, 100 mM sodium acetate) and centrifugation at 15,000 × *g* for 1 min. DNA was eluted in 3 ml of Tris-EDTA (TE) buffer, and 1 g of CsCl was added per ml of DNA solution.

DNA fractions were resolved by equilibrium centrifugation (265,000 × *g*, 16 h, 20°C) in CsCl-ethidium bromide density gradients (13-by-51-mm polyallomer tubes; Beckman). A single DNA band was observed from the soil exposed to $^{12}CH_4$, whereas an additional faint "smear" of more dense DNA was observed up to 1 cm below the bright band from the soil exposed to $^{13}CH_4$ (Fig. 1). Assimilation of both ^{13}C- and ^{12}C-labeled substrates would result in intermediate-density DNA, as observed in the ^{13}C-DNA gradient. The most dense DNA fraction (lower 0.25 cm; ca. 0.35 ml) from the ^{13}C gradient (^{13}C-DNA) and all DNA from the ^{12}C gradient (^{12}C-DNA) were collected with a syringe and needle (19 gauge). The ^{13}C-DNA fraction was subjected to a second ultracentrifugation to remove any small amounts of coextracted ^{12}C-DNA (not visible), providing highly enriched ^{13}C-DNA for PCR. DNA fractions were extracted three times with an equal volume of 1-butanol, dialyzed against TE buffer, ethanol precipitated, and dissolved in 100 μl of TE buffer.

PCR amplification, cloning, sequencing, and analysis. The ^{13}C-DNA and ^{12}C-DNA fractions were used as a template for

TABLE 1. PCR amplification primers used to characterize the ^{13}C- and ^{12}C-DNA fractions

Amplified gene(s)	Primers	Product (bp)	Library constructed[a]	Reference
16S rRNA of *Bacteria*	Eubac27F 1492R	1,450	^{13}C-DNA (100) ^{12}C-DNA (50)	13
16S rRNA of *Archaea*	Arch21F 1492R	1,450	NA[b]	13
18S rRNA of *Eucarya*	EukF EukR	1,500	NA	13
pmoA and *amoA* (putative active site subunit of pMMO and AMO)	A189 A682	525	^{13}C-DNA (50) ^{12}C-DNA (50)	23
pmoA (pMMO active site subunit)	A189 mb661	500	^{13}C-DNA (50)	11
mxaF (MDH active site subunit)	mxa f1003 mxa r1561	550	^{13}C-DNA (50)	30
mmoX (sMMO active site subunit)	mmoX F356 mmoX R898	350	^{13}C-DNA (10)	16

[a] DNA template used for PCR amplification and number of clones in the library (in parentheses).
[b] NA, not applicable.

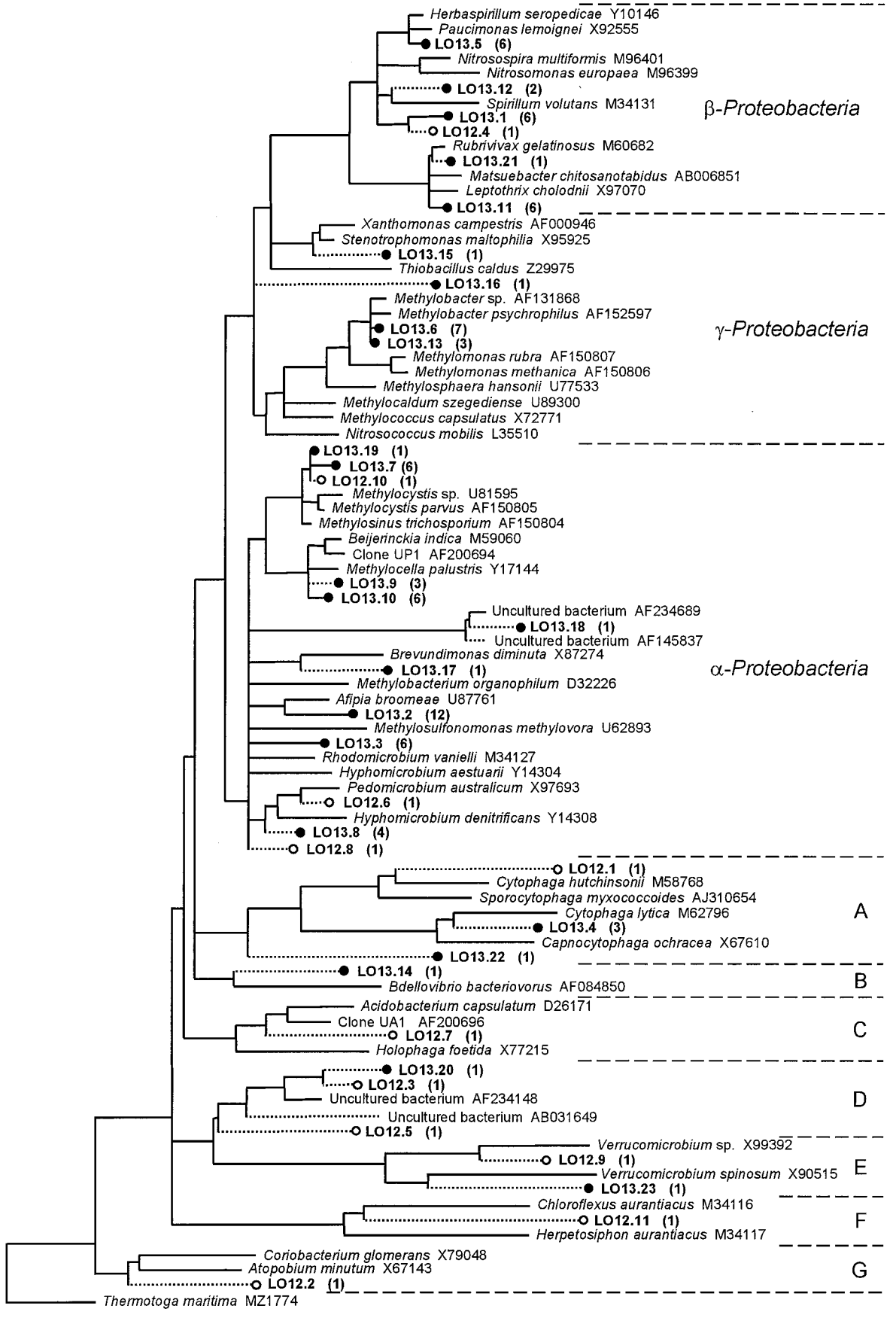
PCR. Primers (Table 1) specific for bacterial (Eubac27F and 1492R), archaeal (Arch21F and 1492R), and eucaryal small-subunit rRNA genes (EukF and EukR) (13) were used to determine the active methanotroph population at the domain level. With ^{12}C-DNA as the template, PCR products of the expected size were obtained with primers that amplified *Bacteria*, *Archaea*, and *Eucarya* small-subunit rRNA genes. However, with ^{13}C-DNA, PCR products were only obtained with bacterial 16S ribosomal DNA (rDNA) primers, indicating that $^{13}CH_4$ was incorporated into a restricted bacterial community. Specific products of the expected size were amplified from both the ^{13}C- and the ^{12}C-DNA fractions with primers encoding key enzymes in CH_4 metabolism (Table 1).

Amplification products were cloned with the TOPO-TA cloning kit (Invitrogen). Libraries were constructed from the ^{13}C-DNA fraction for the bacterial 16S rDNA (100 clones), *pmoA*-A189/A682 (50 clones), *pmoA*-A189/mb661 (50 clones), *mxaF* (50 clones), and *mmoX* (10 clones) PCR products. Libraries were also constructed by using the 16S rDNA (50 clones) and *pmoA*-A189/A682 (50 clones) PCR products from the ^{12}C-DNA fraction. Clones were grouped into operational taxonomic units (OTUs) by restriction fragment length polymorphism (RFLP) analysis by using restriction endonucleases as follows: *Eco*RI/*Rsa*I and *Eco*RI/*Sau*3A for 16S rDNA clones, *Eco*RI/*Rsa*I and *Eco*RI/*Pvu*II/*Hin*cII for *pmoA* clones, and *Eco*RI/*Hin*cII and *Eco*RI/*Rsa*I for *mxaF* clones.

For the 16S rDNA library, full sequence data (ca. 1,450 bp) between *Escherichia coli* positions 8 and 1511 (6) were obtained for one clone from each OTU containing five or more clones. A partial sequence (positions 375 to 890) was determined for each OTU that contained two to five clones and for selected OTUs with single clones. Complete sequence information (between the PCR primers) was obtained for each OTU in the *pmoA* libraries, eight *mxaF* clones, and ten *mmoX* clones. To verify that each unique restriction pattern represented a single clone type, partial sequence data were obtained for at least 10% of the clones within each OTU.

The ARB package (http://www.mikro.biologie.tu-muenchen.de) was used for sequence alignment and phylogenetic analysis. 16S rDNA sequences were aligned by using the ARB automatic alignment tool (Aligner v2.0) and corrected according to secondary structural constraints. Functional gene sequences were aligned manually to sequences from GenBank, and deduced amino acid sequences were used for the analyses. To evaluate tree topology, phylogenies were reconstructed by using evolutionary distance (DNA, Jukes and Cantor model; amino acids, Dayhoff PAM model), maximum parsimony (default parameters for ARB and DNAPARS or ARB and PROTPARS), and maximum likelihood (default parameters for ARB and fastDNAml or ARB and Protein_ML) analyses in conjunction with various filters and sequence subsets (28). Similarities between 16S rDNA sequences were determined by using the similarity matrix option within ARB. No chimeric 16S rDNA sequences were indicated with the Chimera Check program version 2.7 (http://rdp.cme.msu.edu). Inspection of the DNA and amino acid alignments of functional gene sequences did not identify any potential chimeric sequences.

FIG. 2. Maximum-likelihood tree of 16S rDNA sequences amplified from ^{13}C-DNA (●) or ^{12}C-DNA (○) fractions. From the ^{13}C-DNA library (100 clones), 23 OTUs (containing 80 clones) were sequenced, whereas the ^{12}C-DNA library (50 clones) contained 49 OTUs (11 sequenced). The number of clones assigned to each sequenced OTU by RFLP analysis is shown in parentheses. OTU sequences were added to the tree of almost full-length sequences (>1,300 bp) in ARB by using maximum-parsimony analysis. Sequences most closely affiliated to each OTU, as well as sequences (>1,300 bp) of known methanotrophs and nitrifiers, were selected. Partial sequences (<1,300 bp) were chosen from the ARB or GenBank databases when no closely related full-length sequences were available. The tree was constructed by using *Thermotoga maritima* as an outgroup and a 50% conservation filter (962 nucleotides; this excludes positions of ambiguity, missing data, and positions where the frequency of a nucleotide occurring was <50% [28]). Multifurcations indicate branch points where the topology between maximum-likelihood, evolutionary-distance, and maximum-parsimony analyses of the data set were not supported by a strict consensus rule (28). With the exception of LO12.5, LO12.3, and LO13.20, the various treeing analyses consistently recovered all sequences in the groups depicted. Sequences of <1,300 bp (dashed line) were added to the tree by using a maximum-parsimony option within ARB. (A) *Cytophagales*; (B) δ-*Proteobacteria*; (C) *Acidobacterium-Holophaga* group; (D) uncultivated group; (E) *Verrucomicrobia*; (F) green nonsulfur group; (G) *Actinobacteria*.


Herbaspirillum seropedicae Y10146
Paucimonas lemoignei X92555
LO13.5 (6)
Nitrosospira multiformis M96401
Nitrosomonas europaea M96399
LO13.12 (2)
Spirillum volutans M34131
LO13.1 (6)
LO12.4 (1)
Rubrivivax gelatinosus M60682
LO13.21 (1)
Matsuebacter chitosanotabidus AB006851
Leptothrix cholodnii X97070
LO13.11 (6)
β-Proteobacteria
Xanthomonas campestris AF000946
Stenotrophomonas maltophilia X95925
LO13.15 (1)
Thiobacillus caldus Z29975
LO13.16 (1)
Methylobacter sp. AF131868
Methylobacter psychrophilus AF152597
LO13.6 (7)
LO13.13 (3)
γ-Proteobacteria
Methylomonas rubra AF150807
Methylomonas methanica AF150806
Methylosphaera hansonii U77533
Methylocaldum szegediense U89300
Methylococcus capsulatus X72771
Nitrosococcus mobilis L35510
LO13.19 (1)
LO13.7 (6)
LO12.10 (1)
Methylocystis sp. U81595
Methylocystis parvus AF150805
Methylosinus trichosporium AF150804
Beijerinckia indica M59060
Clone UP1 AF200694
Methylocella palustris Y17144
LO13.9 (3)
LO13.10 (6)
Uncultured bacterium AF234689
LO13.18 (1)
Uncultured bacterium AF145837
Brevundimonas diminuta X87274
LO13.17 (1)
α-Proteobacteria
Methylobacterium organophilum D32226
Afipia broomeae U87761
LO13.2 (12)
Methylosulfonomonas methylovora U62893
LO13.3 (6)
Rhodomicrobium vanielli M34127
Hyphomicrobium aestuarii Y14304
Pedomicrobium australicum X97693
LO12.6 (1)
Hyphomicrobium denitrificans Y14308
LO13.8 (4)
LO12.8 (1)
LO12.1 (1)
Cytophaga hutchinsonii M58768
Sporocytophaga myxococcoides AJ310654
Cytophaga lytica M62796
LO13.4 (3)
Capnocytophaga ochracea X67610
A
LO13.22 (1)
LO13.14 (1)
Bdellovibrio bacteriovorus AF084850
B
Acidobacterium capsulatum D26171
Clone UA1 AF200696
LO12.7 (1)
Holophaga foetida X77215
C
LO13.20 (1)
LO12.3 (1)
Uncultured bacterium AF234148
Uncultured bacterium AB031649
LO12.5 (1)
D
Verrucomicrobium sp. X99392
LO12.9 (1)
Verrucomicrobium spinosum X90515
LO13.23 (1)
E
Chloroflexus aurantiacus M34116
LO12.11 (1)
Herpetosiphon aurantiacus M34117
F
Coriobacterium glomerans X79048
Atopobium minutum X67143
LO12.2 (1)
G
Thermotoga maritima MZ1774

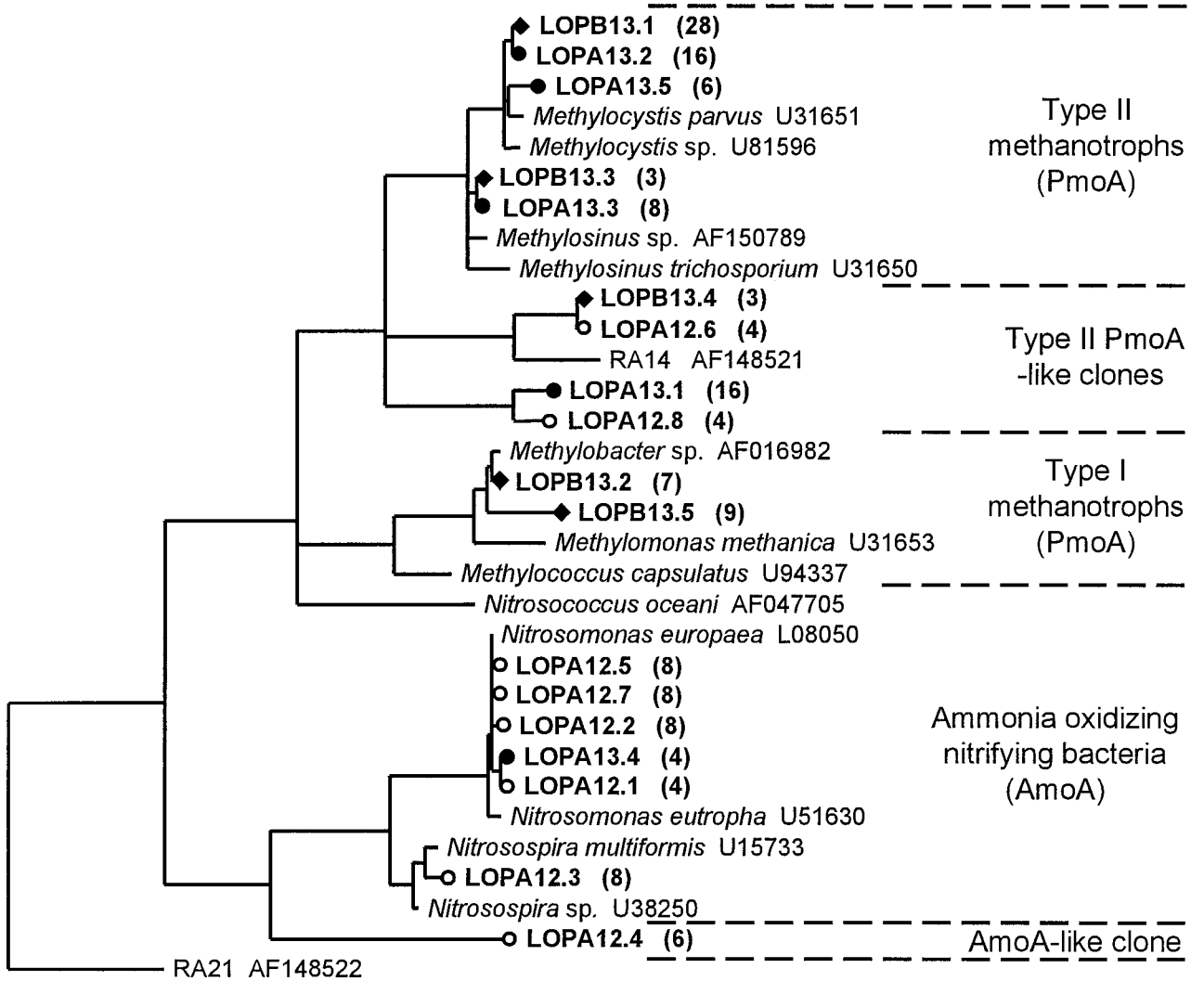


FIG. 3. Maximum-likelihood tree of deduced amino acid sequences of *pmoA* and *amoA* genes amplified from ^{13}C-DNA or ^{12}C-DNA fractions. Clones obtained from the ^{13}C-DNA fraction are prefixed with "LOPA13" when amplified with the A189-A682 (●) primer set or with "LOPB13" when amplified with the A189/mb661 (◆) primer set. Clones obtained from the ^{12}C-DNA fraction and amplified with the A189-A682 (○) primer set are prefixed with "LOPA12." The tree was constructed with a filter (163 aligned amino acid positions; this excludes ambiguities and missing data), and the PmoA sequence of the environmental clone RA21 as an outgroup. Multifurcations indicate branch points where the topology between maximum-likelihood, evolutionary-distance, and maximum-parsimony analyses of the data set were not supported by a strict consensus rule. The number of clones (50 in each library) assigned to each OTU by RFLP analysis is shown in parentheses.

The sequences were deposited in GenBank (AF357990 to AF358055).

16S rDNA libraries. RFLP analysis of 100 16S rDNA clones from the ^{13}C-DNA fraction (Fig. 2) assigned most of the library (70 clones) to 13 OTUs. The remaining 30 clones had unique restriction profiles (30 OTUs), and 10 were partially sequenced. Phylogenetic analysis revealed that, with the exception of five OTUs (containing seven clones), all ^{13}C-DNA clones clustered in the α, β, or γ subclass of the *Proteobacteria* (40, 21, and 12 clones, respectively) (Fig. 2). In stark contrast to the ^{13}C-DNA library, the 50 16S rDNA clones from the ^{12}C-DNA contained 49 unique RFLPs (indicating wide diversity), of which only two were identical to an RFLP from the ^{13}C-DNA. This difference was highlighted by sequencing 10 16S rRNA clones from the ^{12}C-DNA; these were related to a wide variety of *Bacteria* (Fig. 2). One additional clone (LO12.10), with an RFLP identical to LO13.19, was sequenced and identified as an α-proteobacterial methanotroph.

Twenty-six clones in the ^{13}C-DNA library were related to extant methanotrophs (96 to 99% 16S rDNA identity). These OTUs were most similar to the α-proteobacterial methanotrophs *Methylocystis* and *Methylosinus* (LO13.7 and LO13.19) and *Methylocella palustris* (LO13.9 and LO13.10) or to the γ-proteobacterial methanotroph *Methylobacter* (LO13.6 and LO13.13). A further 24 clones were related (92 to 95% identity) to genera in the α subclass of the *Proteobacteria*. Known methylotrophs (*Hyphomicrobium* and *Methylobacterium*) clustered among some (LO13.3, LO13.17, and LO13.8) but not all (LO13.2 and LO13.18) of these OTUs. The third major group of sequences (21 clones; LO13.1, LO13.5, LO13.11, LO13.12,

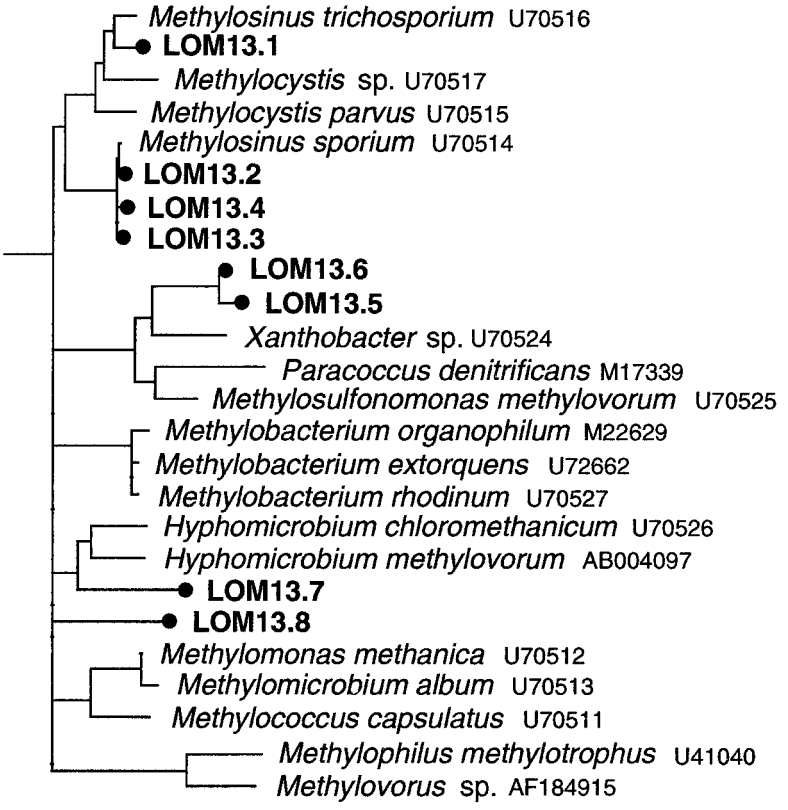


FIG. 4. Maximum-likelihood tree of deduced amino acid sequences of *mxaF* genes amplified from the ^{13}C-DNA fraction (●). The tree was constructed with a filter (171 aligned amino acid positions; this excludes ambiguities and missing data), with the amino acid sequence of the structurally related ethanol dehydrogenase of *Pseudomonas aeruginosa* (26) as an outgroup. Multifurcations indicate branch points where the topology between maximum-likelihood, evolutionary-distance, and maximum-parsimony analyses of the data set were not supported by a strict consensus rule.

and LO13.21) was closely related to genera within the β-*Proteobacteria*. Recovery of clones related to *Bdellovibrio* (LO13.14) and *Cytophaga* (LO13.4 and LO13.22) may have resulted from turnover of ^{13}C due to predation (29, 39). The remaining sequenced clones clustered with the γ-*Proteobacteria* (LO13.15 and LO13.16), a group of uncultivated bacteria (LO13.20), and the *Verrucomicrobia* (LO13.23).

Functional gene libraries. Libraries of 50 *pmoA* clones were constructed with the A189-A682 primer set for both the ^{13}C-DNA and the ^{12}C-DNA fractions. RFLP and sequence analysis of the ^{13}C-DNA library identified three OTUs (LOPA13.2, LOPA13.3, and LOPA13.5) that were similar to PmoA sequences of *Methylocystis* and *Methylosinus* (Fig. 3). Other clones formed a distinct group related to PmoA of type II methanotrophs (LOPA13.1), and four clones were similar to AmoA of *Nitrosomonas europaea* (LOPA13.4).

Analysis of the corresponding ^{12}C-DNA library further demonstrated the different population within the ^{13}C-DNA fraction. Thirty-six clones were assigned to OTUs closely related to AmoA of *Nitrosomonas* (LOPA12.1, LOPA12.2, LOPA12.5, and LOPA12.7) or *Nitrosospira* (LOPA12.3). OTU LOPA12.4 contained a distinct AmoA-like sequence. The remaining OTUs in the ^{12}C-DNA library (LOPA12.6 and LOPA12.8) formed distinct groups that were related to the sequence of the environmental clones RA14 (24) and LOPA13.1, respectively (Fig. 3).

Since type I methanotroph sequences were detected in the 16S rDNA analysis of the ^{13}C-DNA fraction, but not in the corresponding *pmoA* library with the A189-A682 primer set, we also used the methanotroph-specific primer set A189-mb661. Thirty-one clones (LOPB13.1 and LOPB13.3) contained a PmoA that was similar to that of type II methanotrophs (Fig. 3). A further 16 clones (LOPB13.2 and LOPB13.5) clustered with the PmoA of type I methanotrophs. The remaining OTU (LOPB13.4) was identical to LOPA12.6 and most closely related to the environmental clone RA14.

The 50 *mxaF* clones from the ^{13}C-DNA grouped into 32 OTUs and, of the 20 clones sequenced, only 8 showed identity to MxaF. Phylogenetic analysis indicated that four clones (LOM13.1 to LOM13.4) clustered with the MxaF of type II methanotrophs and three (LOM13.5 to LOM13.7) clustered with other α-proteobacterial methylotrophs (Fig. 4). The different analyses of the *pmoA* and *mxaF* data sets consistently recovered the clades depicted in Fig. 3 and 4. Of the 10 *mmoX* clones from the ^{13}C-DNA fraction, six different sequences were identified, all of which were similar to MmoX of the type II methanotroph *Methylosinus* (data not shown).

Characterization of the population that assimilated ^{13}C. In our study, ^{13}C was incorporated into the DNA of metabolically active bacteria that used $^{13}CH_4$ as a sole source of carbon (as shown by the extra, more dense, DNA fraction in the ^{13}C-DNA gradient). Interestingly, the yield of ^{13}C-DNA was very low

relative to that of a methanotroph grown on 1.6 mmol of CH_4 (not shown), suggesting cooxidation of CH_4 in the peat, rapid turnover of methanotroph biomass, or CH_4 production (not observed). Thus, it is probable that a variety of ^{13}C-labeled compounds were produced during the 40-day incubation, including intermediates or products of methanotroph metabolism. Such ^{13}C-labeled compounds could subsequently be assimilated by organisms other than methanotrophs. However, production of the corresponding ^{12}C-labeled compounds (e.g., $^{12}CO_2$) and other trophic interactions would considerably dilute many non-primary substrates within the complex environment of a soil microcosm. Furthermore, since identical centrifugation conditions separated the ^{13}C-DNA and ^{12}C-DNA fractions of a pure methanotroph culture grown on $^{13}CH_4$ or $^{12}CH_4$ by 1 cm (data not shown), we estimate that the ^{13}C-DNA fraction collected (Fig. 1) contained between 75 and 100% ^{13}C. For DNA to possess such a high ^{13}C content and buoyant density, any non-methanotrophs must have been in very close association with the primary $^{13}CH_4$ oxidizers. Therefore, it is most likely that the DNA collected in the "heavy" ^{13}C-DNA fraction originated from microorganisms that assimilated ^{13}C-labeled compounds as a primary carbon source.

Analysis of 16S rRNA and functional genes amplified from the ^{13}C-DNA identified a high proportion of clones that were closely related to extant methanotrophs. These results indicate that a variety of methanotrophs had actively assimilated CH_4 in the peat soil microcosm. The 16S rDNA similarity between LO13.10 and the nearest extant methanotroph, *Methylocella palustris* (12), was <97%, which suggested that a novel methanotroph species was involved in CH_4 oxidation in the microcosm. Since PmoA/AmoA phylogeny of extant strains reflects that obtained with 16S rDNA sequences (23), the *pmoA* libraries support the activity of both type I and type II methanotrophs in the microcosm. The PmoA-like clones in the ^{13}C-DNA fraction (LOPA.13.1 and LOPB13.4) also suggest that an unusual methanotroph within the α subclass of *Proteobacteria* was active in the peat soil. Recent studies of aerobic $^{13}CH_4$ or $^{14}CH_4$ oxidation have detected small amounts of uncharacteristic lipid fractions, which also suggests that unusual methanotrophs are active in soils and sediments (3, 8, 24, 38).

The distribution of clones in the libraries from the ^{13}C-DNA fraction must be interpreted with great caution due to biases inherent in DNA extraction and PCR (43). However, one striking and unexpected feature of the 16S rDNA library is the large number of clones in OTUs that are not closely related to extant methanotrophs (Fig. 2). It is likely that these organisms had assimilated a high proportion of ^{13}C into their DNA, even though the identity of the ^{13}C substrate is unclear. Bacteria capable of growth on methanol and other excreted organic compounds copurify during attempts to isolate methanotrophs into culture (17). Indeed, 22 clones (LO13.2, LO13.3, and LO13.8) in the 16S rDNA library and 3 clones (LOM13.5 to LOM13.7) in the *mxaF* library were sufficiently related to sequences of *Hyphomicrobium*, *Pedomicrobium*, *Rhodomicrobium*, and *Xanthobacter* spp. to suggest that some methylotrophs within the microcosm may have assimilated $^{13}CH_3OH$ excreted by CH_4-oxidizing bacteria.

The other major group of 16S rDNA sequences (LO13.1, LO13.5, LO13.11, LO13.12, and LO13.21) identified in the ^{13}C-DNA library clustered within the β subclass of *Proteobacteria*. No functional genes indicative of β-proteobacterial methylotrophs were identified, a result that is similar to those of other environmental surveys done with *mxaF* PCR primers (18, 19, 21, 30, 37) and may be due to a primer bias. Unfortunately, therefore, it remains unclear which ^{13}C substrate these organisms had assimilated in the peat soil microcosm. Isolates of β-proteobacterial methylotrophs are relatively rare (5, 40), and therefore their diversity might be underdescribed. Our data suggest that some β-*Proteobacteria* are actively involved in the cycling of carbon after CH_4 oxidation in soil. These may represent novel methylotrophs, distinct from most extant strains within the α and γ subclasses of *Proteobacteria*. This ability of SIP to characterize the community in the ^{13}C-DNA fraction with phylogenetic and functional gene PCR primers makes it a powerful technique for resolving microbial structure-function relationships in complex environments such as soil.

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