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Different Atmospheric Methane-Oxidizing Communities in European Beech and Norway Spruce Soils▽†

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Norway spruce (*Picea abies*) forests exhibit lower annual atmospheric methane consumption rates than do European beech (*Fagus sylvatica*) forests. In the current study, *pmoA* (encoding a subunit of membrane-bound CH_4 monooxygenase) genes from three temperate forest ecosystems with both beech and spruce stands were analyzed to assess the potential effect of tree species on methanotrophic communities. A *pmoA* sequence difference of 7% at the derived protein level correlated with the species-level distance cutoff value of 3% based on the 16S rRNA gene. Applying this distance cutoff, higher numbers of species-level *pmoA* genotypes were detected in beech than in spruce soil samples, all affiliating with upland soil cluster α (USCα). Additionally, two deep-branching genotypes (named 6 and 7) were present in various soil samples not affiliating with *pmoA* or *amoA*. Abundance of USCα *pmoA* genes was higher in beech soils and reached up to $(1.2 \pm 0.2) \times 10^8$ *pmoA* genes per g of dry weight. Calculated atmospheric methane oxidation rates per cell yielded the same trend. However, these values were below the theoretical threshold necessary for facilitating cell maintenance, suggesting that USCα species might require alternative carbon or energy sources to thrive in forest soils. These collective results indicate that the methanotrophic diversity and abundance in spruce soils are lower than those of beech soils, suggesting that tree species-related factors might influence the *in situ* activity of methanotrophs.

Methanotrophic bacteria in aerated soils contribute significantly to the global flux of methane (CH_4) and consume about 30 Tg $year^{-1}$ of atmospheric CH_4 (12). Forest soils are considered to be the most effective sinks of CH_4 among these soils (8, 48). Atmospheric CH_4 oxidation and the methanotrophic communities in forest soils may be affected by various abiotic factors, e.g., pH, water content, and soil temperature (32).

Cultivated methanotrophs belong to the families *Methylocystaceae* and *Beijerinckiaceae* (both *Alphaproteobacteria*), *Methylococcaceae* (*Gammaproteobacteria*), and the recently proposed *Methylacidiphilaceae* (*Verrucomicrobia*) (43). Most of these cultivated species are restricted to one-carbon compounds as carbon and energy sources (53). Only *Methylocella* species may grow on multicarbon compounds (9). Isolates of *Methylocystis* (i.e., strains LR1, DWT, and SC2) utilize atmospheric CH_4 (1.8 ppm by volume [ppmv]); however, growth is not apparent at this concentration (3, 16, 28).

Genes of the CH_4 monooxygenase (MMO) are well-established functional markers of methanotrophs (38). *pmoA* encodes the hydroxylase of membrane-bound CH_4 monooxygenase (pMMO) while *mmoX* encodes the corresponding subunit of cytoplasmic CH_4 monooxygenase (sMMO). Facultative methanotrophic species of *Methylocella* exclusively possess sMMO and thus will not be detected in *pmoA* analyses (10, 38). Atmospheric CH_4-oxidizing communities have been analyzed in a variety of forest soils, and *Methylocystis* species and upland soil cluster α (USCα)-affiliated genotypes are detected in most of these soils (32). Methanotrophs affiliated with USCγ and clusters 1 and 5 are also abundant or even dominant at certain forest sites (30, 31, 33). Numbers of methanotrophs in forest soil range from 0.4×10^6 to 21×10^6 cells per g of dry weight (g_{DW}) as determined by quantitative PCR (qPCR) (29, 33), and these bacteria have resisted cultivation (32).

Coniferous forests can have lower annual atmospheric CH_4 uptake rates than deciduous forests (6, 40). European beech (*Fagus sylvatica*) soils exhibit a lower capacity to oxidize CH_4 than Norway spruce (*Picea abies*) soils, indicating a causative effect of tree species on methanotrophic community (11). In the current study, the methanotrophic community composition and abundance of methanotrophs in soil from forests with adjacent beech and spruce stands were assessed to evaluate the potential effect of tree species on methanotrophic communities.

MATERIALS AND METHODS

Study sites and sampling. Soil samples were taken from three temperate forests with European beech (*F. sylvatica*) and adjacent Norway spruce (*P. abies*) stands in Germany, i.e., Solling, Steigerwald, and Unterlüß. Soils at any sampling site were classified as dystric cambisols (11). Five soil cores were taken at each of the six sites and stored in open plastic bags at 5°C for up to 4 days until they were separated into depth layers, manually homogenized, and frozen at −80°C. Soil pH values were similar at beech and spruce sites and ranged from 3.9 to 5.4 (11). Samples from the O_a horizon (blackish humic layer) and from mineral soil at a depth of 0 to 5 cm (0- to 5-cm soil) were pooled and used for molecular community analyses since these layers exhibited the highest atmospheric CH_4 oxidation rates (11).

DNA extraction. DNA was extracted from 0.3 g of frozen soil (0.1 g of O_a horizon plus 0.2 g of 0- to 5-cm mineral soil) according to the protocol of Stralis-Pavese and colleagues (51) by mechanical, chemical, and enzymatic lysis. The following four modifications to the protocol were made. (i) Lysis buffer I used for initial extraction of soil also contained 20 mg ml^{-1} of polyvinyl pyrrolidone. (ii) Lysis buffer II for reextraction of soil samples was identical with

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buffer I. However, polyvinyl pyrrolidone and lysozyme were not added. (iii) The DNA pellet was dried for 15 min at 40°C after the washing step. (iv) DNA was eluted with Tris-HCl (pH 8.0). DNA extracts were quantified using a Quant-iT PicoGreen dsDNA kit (Invitrogen, Germany). For each of the 30 soil cores, DNA was extracted in triplicate, i.e., 90 extracts in total.

PCR and cloning. Gene libraries of *pmoA*, *mmoX*, and *Verrucomicrobia*-related *pmoA* were set up. DNA extracts in triplicate per soil sample were pooled, and 1 ng of DNA was used as a template in PCRs. PCR premix F (Epicentre) combined with an Invitrogen *Taq* polymerase recombinant (Invitrogen, Germany) was used. *pmoA* was amplified with primers A189f (GGN GAC TGG GAC TTC TGG) and A682r (GAA SGC NGA GAA GAA SGC) (500 nM) (20). The initial denaturation step was at 94°C for 5 min, and the final elongation was at 72°C for 10 min. Denaturation and elongation during repeated PCR cycles was at 94°C (1 min) and 72°C (1 min), respectively. An 11-cycle touchdown was performed with a stepwise decrease of the annealing temperature from 65°C to 55°C (1 min), followed by 24 cycles with an annealing temperature of 55°C (1 min). *Taq* DNA polymerase was added after the initial denaturation step. *mmoX* was amplified with primers mmoX206f (ATC GCB AAR GAA TAY GCS CG) and mmoX886r (ACC CAN GGC TCG ACY TTG AA) (500 nM) (22). Five precycles with an annealing temperature of 56°C were performed, followed by 35 cycles at 59°C. Genomic DNA of *Methylococcus capsulatus* was used as positive control for *pmoA*- and *mmoX*-specific PCRs. Four novel primers were designed to prove the presence of methanotrophic *Verrucomicrobia* in the soil samples, i.e., Verruco29f (5′-AAG AYM GRA TGT GGT G-3′), Verruco120f (5′-GCC YAT AGG WGC RAC MT-3′), Verruco391r (5′-GTC CAT AGT ATT CCA C-3′), and Verruco500r (5′-ACD CCH CCN GCA AAR CT-3′). PCR was performed with pooled soil DNA extracts from all soil cores of the six sites (i.e., beech and spruce of Solling, Steigerwald, and Unterlüß). Since no positive control for *Verrucomicrobia*-related *pmoA* was available, PCR was performed with different primer combinations and annealing temperatures from 50°C to 70°C, with and without the addition of 0.12% bovine serum albumin (BSA), and as simple or nested assays.

PCR products were purified by agarose gel electrophoresis, and bands of the right size were excised and purified with a Montage Gel Extraction Kit (Millipore). Equal concentrations of all five PCR products of one sampling site were pooled and cloned by AGOWA (Germany). Per sampling site, 48 to 96 clones were sequenced by MacroGen (South Korea).

Sequence data analysis. 16S rRNA gene and *pmoA* gene sequences from 22 pure cultures were retrieved from GenBank (5). The gene fragments corresponded to regions amplified by the commonly used primer pair A189f and A682r (20) and the pair 27F and 1492R (35), respectively, and were further analyzed in ARB (36). Gene fragments were aligned, and *pmoA* sequences were *in silico* translated. Pairwise comparisons of all *pmoA* (DNA), PmoA (amino acids), and 16S rRNA gene sequences were performed to calculate the nucleotide or amino acid difference, *D* (18). The percentage sequence similarity of *pmoA* and PmoA sequence pairs was plotted versus the percentage sequence similarity of the 16S rRNA gene sequence pairs of the same strains. The similarity, *S*, was calculated according to the following equation: $S = 1 - D$.

pmoA sequences retrieved from the six *pmoA* gene libraries of the sampled sites were aligned and *in silico* translated. Amino acid sequences were analyzed with the programs DOTUR (46) and ∫-LIBSHUFF (47) using an uncorrected distance matrix as input data set. The number of operational taxonomic units (OTUs) achieved by DOTUR was calculated for a 7% distance, which is an estimated cutoff value for methanotrophic species-level OTUs (Fig. 1). Coverage, *C*, of *pmoA* gene libraries was calculated according to the following equation, where *n* is the number of OTUs with only one sequence (singletons), and *N* is the number of clones examined: $C = [1 - (n/N)] \times 100$.

qPCR assays targeting detected genotypes. *Bacteria* were quantified with a modified assay established by Zaprasis and coworkers (55) using primers Eub341f (CCT ACG GGA GGC AGC AG) and Eub534r (ATT ACC GCG GCT GCT GG) (42). New qPCR assays (Q-USCα, Q-C7, and Q-AOB) were developed to assess the abundance of *pmoA* genes of USCα, *pmoA* and *amoA* genes of cluster 7 (C7), and *amoA* genes of ammonium oxidizing bacteria [AOB] within the *Betaproteobacteria*) detected in the soils. The following novel primers were designed and used in combination with common published primers (Table 1): USCα-346f (5′-TGG GYG ATC CTN GCN C-3′), C7-128r (5′-CCA ATG GGG AGC CTA AAT-3′), amoA38f (5′-AAT GGT GGC CGG TKG TNA C-3′), and amoA200r (5′-GAC CAC CAG TAR AAD CCC C-3′). The establishment of a primer system for the specific quantification of cluster 6 did not succeed; i.e., primers yielded unspecific by-products. Specificity of the novel qPCR assays was evaluated by means of nontarget DNA that had at least two mismatches at the binding site of the target group-specific primers. *pmoA* fragments of the different detected genotypes and of *M. capsulatus* (NCIMB 11853) were used as nontargets. Dilution series of target DNA quantified with a Quant-iT PicoGreen double-stranded DNA (dsDNA) kit (Invitrogen, Germany) were applied as quantitative calibration standards. A 16S rRNA gene fragment of *Escherichia coli* amplified with primers 27f and 907mr (35) was used as standard DNA for the *Bacteria*-targeting assay. For Q-USCα, Q-C7, and Q-AOB, an M13 PCR product of a respective clone insert for *pmoA* and *amoA* was used.

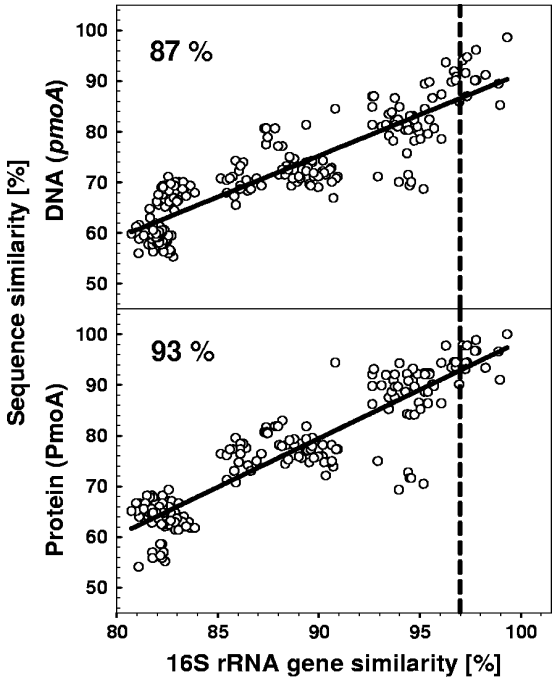

FIG. 1. Correlation of DNA and protein sequence similarity of *pmoA* and PmoA versus 16S rRNA gene similarity (22 pairwise comparisons of methanotrophic strains). The dashed line represents the 97% species cutoff value based on the 16S rRNA gene (49). Percentage values indicate the similarity cutoff value for species-level OTUs based on *pmoA* and PmoA sequence corresponding to distance values of 13% and 7%, respectively (intersection of dashed and regression line). R^2 was 0.85 for the DNA panel and 0.79 for the protein panel. Accession numbers of analyzed sequences are given in Table S1 in the supplemental material.

All PCRs were performed in triplicate in 20-μl volumes using Thermosprint 96 PCR plates sealed with Thermosprint transparent sealing tapes (Bilatec, Germany). Five microliters of DNA template, i.e., 1 to 6 ng of DNA, were added to 15 μl of Master Mix containing the primers and the iQ SYBR Green Supermix (Bio-Rad, Germany). Final primer concentrations were 1,000 nM for Q-AOB and 500 nM for Q-USCα, Q-C7, and the *Bacteria*-targeting assay. The initial denaturation step was at 95°C for 8 min. Denaturation and elongation during PCR was at 95°C (30 s) and 72°C (15 s for Q-USCα and Q-C7, 20 s for Q-AOB, and 25 s for the *Bacteria*-targeting assay), respectively. Fluorescence data were acquired during the elongation step at 72°C since primer dimer formation was not evident by melting curve analysis. More details on the assays are presented in Table 1. qPCR was performed on an iCycler thermocycler with an iQ5 multicolor real-time PCR detection system. Data were analyzed with iQ5 optical system software (version 2.0; Bio-Rad, Germany).

Target group specificity of the assay Q-USCα was further evaluated by cloning (TOPO TA Cloning Kit; Invitrogen, Germany) purified (PCR purification kit; Qiagen, Germany) qPCR products derived from soil extracts and subsequent sequencing of 10 clone insert sequences. All retrieved sequences were *pmoA* genes and affiliated with USCα (data not shown).

Correction of qPCR measurements affected by coextracted inhibitory compounds. The assay INHIB-CORR using primers T7-Prom (TAA TAC GAC TCA CTA TAG GG, Promega, Germany) and M13r (CAG GAA ACA GCT ATG ACC) (41) was established to calculate a factor correcting for PCR inhibition in each DNA extract caused by coextracted inhibitory compounds, such as humic substances (54). The vector region of the plasmid pCR 2.1-TOPO (In-

TABLE 1. Conditions of qPCR assays

Assay	Primers	Primer concn (nM)	Amplicon length (bp)	Annealing		No. of PCR cycles	Efficiency (%)[a]	Detection limit (no. of genes per reaction)	Reference or source
				Temp (°C)	Time (s)				
Bacteria-targeting	Eub341f/Eub534r	250	193	55.7	25	35	97	10^2	55
Q-USCα	USCα-346f/A682r	500	185	57.3	15	38	85	10^1	This study
Q-AOB	amoA38f/amoA200r	1,000	164	50.0	20	40	87	10^2	This study
Q-C7	A189f/C7-128r	500	128	61.2	15	38	94	10^1	This study
INHIB-CORR[b]	T7-Prom/M13r	500	201	61.2	15	38	88	10^2	This study

[a] Amplification efficiency as calculated by the iQ5 optical system software (version 2.0; Bio-Rad, Germany).
[b] The assay was established to determine the quantitative degree of inhibition in qPCR measurements due to coextracted inhibitory compounds, such as humic acids.

vitrogen, Germany) without a DNA insert amplified with primers M13f and M13r (41) served as a target for the assay INHIB-CORR and was used as inhibition control DNA. Every DNA extract was spiked with 0.2×10^4 inhibition control DNA molecules μl^{-1}. Spiked DNA did not affect the amplification efficiency of the genotype-specific assays Q-USCα, Q-C7, and Q-AOB, and the *Bacteria*-targeting assay (data not shown). Final primer concentrations of the assay INHIB-CORR were 500 nM, and elongation during PCR was performed for 15 s. Further details are given in Table 1.

Spiked environmental DNA extracts were measured with genotype-specific assays and with INHIB-CORR. The logarithmic values of the starting quantity measured with the genotype-specific assays [$lg(SQ)_{measured}$] were corrected for the inhibition factors determined with INHIB-CORR and for the difference in amplification efficiency (E) between the genotype-specific assays and INHIB-CORR (equation 1). Spiked DNA extracts were diluted to keep inhibition factors below a factor of 2. Corrected logarithmic values of the starting quantity [$lg(SQ)_{corrected}$] were then converted to gene numbers per ng of DNA (ng_{DNA}^{-1}):

$$lg(SQ)_{corrected} = lg(SQ)_{measured} \times [lg(SQ)_{INHIB\text{-}CORRset}/lg(SQ)_{INHIB\text{-}CORRis}] \times (E_{Assay}/E_{INHIB\text{-}CORR}) \quad (1)$$

For comparison of USCα gene abundances measured using the new Q-USCα assay with those measured using the assay FOREST (34) in a previous work (33), both assays were applied to the same DNA extracts. *In silico* coverage and measured numbers of USCα genes were compared (see Table S2 in the supplemental material).

Nucleotide sequence accession numbers. *pmoA* and *amoA* sequences were deposited at the European Molecular Biology Laboratory (EMBL) database under accession numbers FN564572 to FN564934.

RESULTS

***pmoA*- and PmoA-based distance cutoff values for estimating species-level OTUs of methanotrophs.** Sequence similarities of *pmoA*s and PmoAs and 16S rRNA genes of 22 methanotrophic isolates were linearly correlated, as suggested previously (19). This linear correlation was the basis to define distance cutoff values for methanotrophic species-level OTUs. The 97% species cutoff value based on 16S rRNA genes (49) corresponded with similarity cutoff values of 13% and 7% at the DNA and protein levels, respectively, for methanotrophic species-level *pmoA*-based OTUs (Fig. 1). Ten species-level USCα-affiliated OTUs (USCα 1 to 10) were resolved when these species-level cutoff values were used to analyze all available sequences (i.e., those published and those obtained in the current study) (Fig. 2). Clusters 5 and MHP were composed of nine and five species-level OTUs, respectively. Sequences within cluster AC (aquatic clones) and *Methylocapsa acidiphila*-related sequences formed separated OTUs. USCα and clusters 5 and MHP might represent three methanotrophic genera.

Detected genotypes. *pmoA* sequences were analyzed to resolve the identity of methanotrophic bacteria in pooled O_a horizon and mineral soil samples from the three forests, Solling, Steigerwald, and Unterlüß; 41 to 85 sequences were obtained per sampling site, with a total of 204 and 162 sequences from beech and spruce soils, respectively. Coverage of gene libraries based on the 7% distance cutoff value (Fig. 1) was close to 100% for most gene libraries, indicating sufficient sampling at most sites (Solling beech, 93%; Solling spruce, 82%; Steigerwald beech, 99%; Steigerwald spruce, 100%; Unterlüß beech, 100%; and Unterlüß spruce, 100%). Nine species-level OTUs were retrieved from beech soils (Fig. 3). These sequences affiliated with seven different OTUs within USCα (USCα-1, -2, -6, -7, -8, -9, and -10) and two deep-branching clusters (clusters 6 and 7) (Fig. 3 and 4) that might represent PmoA or AmoA sequences. Cluster 6 includes the environmental sequence EU723753 (retrieved from a forest soil) (26). The novel cluster 7 exclusively contains sequences obtained from Solling beech soil samples. In contrast, only five OTUs were detected in spruce soils (USCα-1, -4, -7, and -8 and cluster 6), indicating that methanotrophic diversity in spruce soils was lower than that in beech soils, as confirmed by statistical analysis (∫-LIBSHUFF, $P < 0.05$) (47). USCα-1 was the most frequently detected OTU associated with both tree species. USCα-1 includes RA14, the first *pmoA* genotype found to be potentially associated with atmospheric methane oxidation (21).

amoA sequences of *Betaproteobacteria* were occasionally found in Solling beech and Unterlüß spruce soils but not in Steigerwald soils. A high proportion of *amoA* sequences were detected in spruce soil from Solling (Fig. 3) and led to a lower coverage of methanotrophic OTUs than in other sampling sites and probably to an underestimation of methanotrophic diversity at this site. *mmoX* and *Verrucomicrobia*-related *pmoA* genes were not detected in any soil sample.

Abundance of methanotrophic, ammonia-oxidizing, and total *Bacteria*. *In silico* coverage with the USCα primers used in the FOREST assay (34) approximated 86% of the USCα genotypes detected in the current study, whereas coverage with the new primers used in the new Q-USCα assay was 98% (see Table S2 in the supplemental material). Q-USCα *pmoA* gene numbers exceeded those detected with FOREST by a factor of up to 8, as revealed from Steigerwald beech soil DNA extracts (see Table S2). Gene numbers of cluster 7 were below the detection limit of the corresponding qPCR assay (8.8×10^{-1} genes ng_{DNA}^{-1}). Abundance of USCα was higher in beech than in spruce soils at all three forest sites (Fig. 5A) ($P > 0.05$). USCα *pmoA* gene numbers ranged from 1.3×10^3 to 3.3×10^3 *pmoA* genes ng_{DNA}^{-1}, i.e., 0.3×10^8 to 1.2×10^8 *pmoA* genes

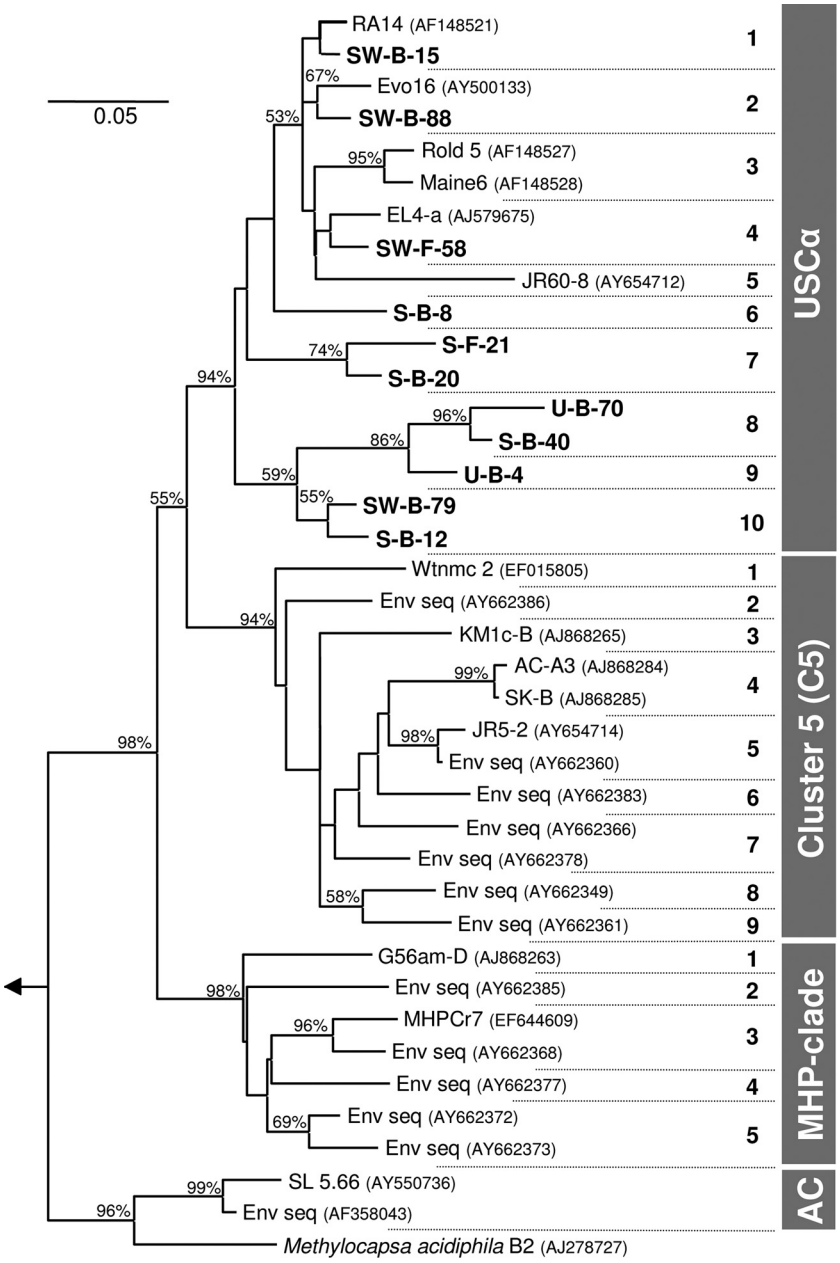

FIG. 2. Dendrogram of species-level OTUs within USCα (30); clusters 5 (31), MHP (peat bog) (7), and AC (aquatic environments; named in this study); and *Methylocapsa acidiphila*-related sequences. Analysis of species-level OTUs was performed with 406 PmoA sequences based on a 7% sequence distance cutoff value using DOTUR (46). Analysis was restricted to amino acids 59 to 206 according to the PmoA of *Methylococcus capsulatus* Bath (NC_002977) and resulted in 26 different species-level OTUs. Thirty-nine selected sequences out of these 26 OTUs were used to construct a dendrogram in MEGA4 (52) using the neighbor-joining method (18) without evolutionary correction to apply the distance cutoff concept (10,000 bootstrap steps) (45). Bootstrap values higher than 50% are shown. Bold names indicate sequences from the current study. Accession numbers are given in parentheses. Bold numbers on the right side (1 to 10) represent different species-level OTUs within the corresponding clusters. The PmoA sequence of *M. capsulatus* Bath (NC_002977) was used as an out-group (arrow). The scale bar represents 5% sequence difference. Env seq, environmental sequence.

g_{DW}^{-1}. *amoA* genes exceeded *pmoA* genes by a factor of 10, ranging from 1.3×10^4 to 3.7×10^4 *amoA* genes ng_{DNA}^{-1}, i.e., 0.3×10^9 to 2.6×10^9 *amoA* genes g_{DW}^{-1} (Fig. 5B). Highest abundance of *amoA* genes was detected in Solling spruce soil samples. Abundance of bacterial 16S rRNA genes was generally higher in spruce soils than in beech soils (Fig. 5C). Gene numbers ranged from 0.4×10^6 to 1.1×10^6 16S rRNA genes ng_{DNA}^{-1}, i.e., 6.3×10^9 to 7.2×10^{10} 16S rRNA genes g_{DW}^{-1}. Assuming that a methanotrophic cell contains two *pmoA* copies (50) and that a bacterial cell contains 4.13 16S rRNA gene copies (27), ratios of USCα to total *Bacteria* cell numbers were up to 2% and 0.5% for beech and spruce soils, respectively.

pmoA numbers and atmospheric CH_4 oxidation rates of the six forest soils (11) were used to estimate atmospheric CH_4 oxidation rates per cell. This calculated cell-specific activity was higher in beech than in spruce soil samples from Unterlüß and Solling, whereas cell-specific activities were similar in beech and spruce soils from Steigerwald (Table 2).

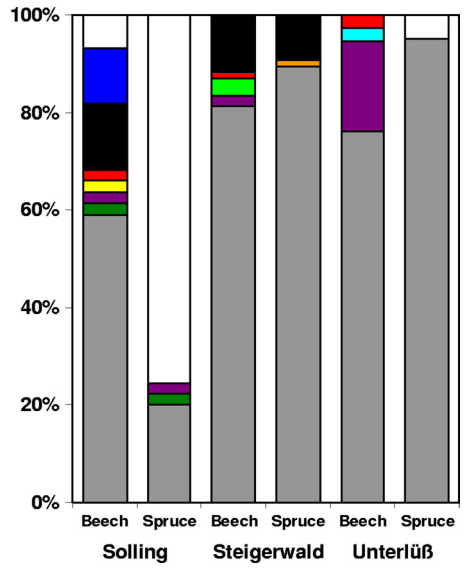

FIG. 3. Community composition based on *pmoA* clone libraries. OTUs were defined according to a methanotrophic species-level cutoff value of 7%. Gene libraries were retrieved from *pmoA* PCR products from beech and spruce soils (O_a horizon pooled with 0- to 5-cm mineral soil) from three forests, Solling, Steigerwald, and Unterlüß. The numbers of analyzed sequences per clone library were as follows: Solling beech, 44; Solling spruce, 45; Steigerwald beech, 85; Steigerwald spruce, 75; Unterlüß beech, 75; and Unterlüß spruce, 41. Analysis was performed with 406 PmoA sequences using amino acids 59 to 206 according to the PmoA sequence of *M. capsulatus* Bath (NC_002977). Gray, USCα-1; dark green, USCα-7; purple, USCα-8; light green, USCα-2; orange, USCα-4; light blue, USCα-9; yellow, USCα-6; red, USCα-10; black, cluster 6; dark blue, cluster 7; white, ammonia-oxidizing bacteria within the *Betaproteobacteria*.

DISCUSSION

USCα is dominant in a variety of forest soils (24, 30, 32, 33). Consistently, *pmoA* sequences most frequently related to USCα were detected in *pmoA* gene libraries of Solling, Steigerwald, and Unterlüß forest soils in the current study. Clone libraries revealed a lower diversity of methanotrophic bacteria in spruce than in beech soils at all three sampling sites (Fig. 3), evidence that tree species affect methanotrophic community structure. USCα-1 was most frequently detected in both beech and spruce soils, indicating that this species-level OTU might constitute a dominant taxon.

Differences mainly occurred in less abundant species-level OTUs. USCα-2, -6, -9, and -10 and cluster 7 were not detected in spruce soils. Coniferous trees release monoterpenes that may reduce CH_4 oxidation (1, 37). Atmospheric CH_4 consumption rates are often lower in coniferous forest soils than in deciduous forest soils (6, 11, 40). USCα-2, -6, -9, and -10 and cluster 7 might represent genotypes that are more sensitive to monoterpenes than USCα-1, -4, -7, and -8 and cluster 6. Tree species did not affect methanotrophic community composition in a boreal tree succession experiment, as revealed by membrane lipid fingerprinting (39). However, the analysis of *pmoA* genes in the current study provides a more refined resolution of genotypes and facilitates the identification of methanotrophs on genus and species levels. Differences observed in the methanotrophic community structure between beech and spruce soils might reflect differences in monoterpene concentrations between coniferous and deciduous forests (37).

Methylocystis or alternative atmospheric CH_4-oxidizing bacteria, such as those belonging to clusters 1 and 5 (32), were not detected in the forest soils of Solling, Steigerwald, and Unterlüß. *mmoX* genes were not detected, indicating that facultative methanotrophic species of the genus *Methylocella* and other sMMO-possessing methanotrophs were either not present or below the detection limit. Methanotrophic *Verrucomicrobia* bacteria were recently isolated from acidic environments (17, 23, 44). It was hypothesized that these bacteria might have been overlooked in other acidic environments due to their divergent *pmoA* gene that cannot be amplified with common *pmoA* primers (17). Nonetheless, *Verrucomicrobia*-like *pmoA* genes could not be amplified in the investigated soils with new

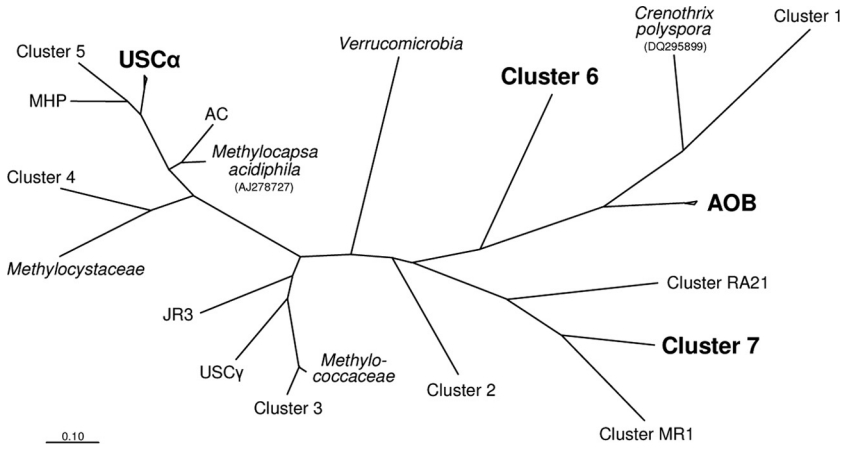

FIG. 4. Phylogenetic tree showing the relationship of PmoA sequences obtained from forest soils in this study to known groups of PmoA and AmoA sequences. USCα (21), USCγ (30), cluster 1 (33), JR3 (2), and *Methylocystaceae* (16) represent putative atmospheric methane oxidizing genotypes. The tree was calculated based on amino acids 59 to 206 according to the PmoA sequence of *M. capsulatus* Bath (NC_002977) using the ARB-implemented PROML method (36) with the evolutionary model JTT (25). The scale bar represents 10% sequence difference. Accession numbers of sequences used for tree reconstruction are as follows: AF148521, AF148522, AF200729, AJ278727, AJ579669, AJ868245, AJ868259, AJ868265, AJ868278, AJ868281, AJ868409, AY550736, DQ295899, EF591085, EF644409, FJ970601, FN564735, FN564878, FN564924, FN564930, U76553, and NC_002977.

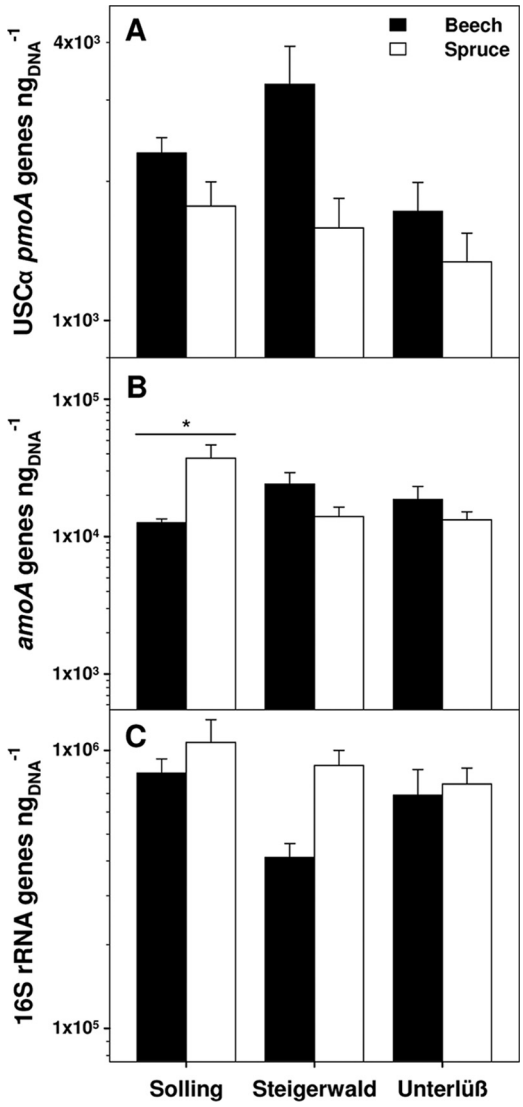

FIG. 5. *pmoA*, *amoA*, and 16S rRNA gene numbers. Bars show the number of *pmoA*, *amoA*, and 16S rRNA genes measured by qPCR in beech and spruce soils from Solling, Steigerwald, and Unterlüß. Error bars indicate standard deviations of three replicate DNA extractions of five soil cores, i.e., from a total of 15 values. The asterisk indicates significant differences between beech and spruce soil (Mann-Whitney U test).

primers that target *pmoA1* and *pmoA2* of *Verrucomicrobia*, suggesting the absence of this taxon. AOB of the genus *Nitrosospira* were highly abundant in Solling spruce soil (Fig. 3 and 5). This forest soil had a 3- to 13-fold higher ammonium content than the other soils (11), which might explain the high proportion of AOB in the gene library. It cannot be excluded that atmospheric CH_4 was oxidized to some extent by AOB since CH_4 is a substrate homolog for ammonia monooxygenase or that CH_4 oxidation activity was lowered by the competitive inhibition of MMO by ammonium in the spruce soil of Solling forest (4, 13).

Abundance of USCα and its relative contribution to the total bacterial community were slightly higher in beech than in spruce soils (Fig. 5). *pmoA* gene numbers ranged from 0.3×10^8 to 1.2×10^8 *pmoA* genes g_{DW}^{-1}, values that exceeded those obtained with the FOREST assay (0.9×10^6 to 6.2×10^6 *pmoA* genes g_{DW}^{-1}) (29, 33). The primers used with the PCR assay FOREST cover only 81% of the available sequences (see Table S2 in the supplemental material), and the new Q-USCα assay yielded 8-fold higher gene numbers than the FOREST assay (see Table S2). Thus, the lower coverage of primers used in the FOREST assay might contribute to the differences in abundances measured in the previous and present study.

Calculated cell-specific CH_4 oxidation rates were slightly higher in beech than in spruce soils (Table 2). A CH_4 oxidation rate of at least 40×10^{-18} mol of CH_4 $cell^{-1}$ h^{-1} may be required for maintenance metabolism (33). Estimated USCα-specific oxidation rates approximated 800×10^{-18} mol of CH_4 $cell^{-1}$ h^{-1}, suggesting that these organisms conserve enough energy for growth during the oxidation of atmospheric CH_4 (33). However, these rates may have been overestimated since *pmoA* gene numbers were likely underestimated with the FOREST assay. Cell-specific CH_4 oxidation rates calculated in the current study ranged from 1×10^{-18} to 14×10^{-18} mol of CH_4 $cell^{-1}$ h^{-1}; these values are lower than required for maintenance of methanotrophic biomass, which raises the question of how atmospheric CH_4 oxidizers are capable of growth in aerated soils (14). CH_4 production was not measured in soil samples from the six sampling sites under anoxic conditions (11), and, thus, it is unlikely that USCα species grow also on CH_4 produced in soil. Another possible survival mechanism would be the utilization of substrates other than or in addition to CH_4 (14). It is known that *Methylocella silvestris*, which was isolated from a forest soil, is able to utilize alternative carbon compounds, such as acetate or methanol (9, 15). This suggests that USCα might rely on additional carbon sources to conserve enough energy for cell maintenance and growth. The capacity to use alternative carbon sources might thus lead to a high relative abundance of USCα (i.e., up to 2% of the total detected bacterial community), as has been observed in this study. Nonetheless, calculations of cell-specific CH_4 oxidation rates are based on the detection of DNA and, thus, on the assumption that all detected genes represented active cells. This assumption might be not fully true and might have led to an underestimation of cell-specific CH_4 oxidation rates.

Soil pH, temperature, and water content may impact community structure of methanotrophs (6, 8, 32). Nonetheless, different CH_4 oxidation rates occur in acidic beech and spruce soils at equal matrix potentials and temperatures, indicating that tree species itself is an important environmental determinant of atmospheric CH_4 consumption in forest soils (11).

TABLE 2. Estimated cell-specific atmospheric CH_4 oxidation rates of USCα

Forest	Cell-specific CH_4 oxidation rate ($\times 10^{-18}$ mol of CH_4 $cell^{-1}$ h^{-1}) by soil type[a]	
	Beech	Spruce
Solling	6.9 ± 1.5	1.5 ± 0.3
Steigerwald	14.0 ± 2.6	14.2 ± 2.1
Unterlüß	4.1 ± 0.7	1.8 ± 0.4

[a] Cell-specific rates are based on *pmoA* cell numbers of USCα assuming two *pmoA* copies per cell (50). Atmospheric CH_4 oxidation rates in O_a horizon and 0- to 5-cm mineral soil (11) were divided by USCα cell numbers.

Monoterpenes have been proposed as factors that lower methanotrophic metabolic activities in spruce soils (1, 37). These collective results indicate that the methanotrophic diversity and abundance in spruce soils are lower than those of beech soils, suggesting that tree species-related factors might influence the *in situ* activity of methanotrophs.

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REFERENCES

1. **Amaral, J. A., and R. Knowles.** 1998. Inhibition of methane consumption in forest soils by monoterpenes. J. Chem. Ecol. **24:**723–734.
2. **Angel, R., and R. Conrad.** 2009. *In situ* measurement of methane fluxes and analysis of transcribed particulate methane monooxygenase in desert soil. Environ. Microbiol. **11:**2598–2610.
3. **Baani, M., and W. Liesack.** 2008. Two isozymes of particulate methane monooxygenase with different methane oxidation kinetics are found in *Methylocystis* sp. strain SC2. Proc. Natl. Acad. Sci. U. S. A. **105:**10203–10208.
4. **Bédard, C., and R. Knowles.** 1989. Physiology, biochemistry, and specific inhibitors of CH_4, NH_4^+, and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev. **53:**68–84.
5. **Benson, D. A., I. K. Karsch-Mizrachi, D. J. Lipman, J. Ostell, and D. L. Wheeler.** 2008. GenBank. Nucleic Acids Res. **36:**D25–D30.
6. **Borken, W., Y. J. Xu, and F. Beese.** 2003. Conversion of hardwood forests to spruce and pine plantations strongly reduced soil methane sink in Germany. Glob. Chang. Biol. **9:**956–966.
7. **Chen, Y., M. G. Dumont, N. P. McNamara, P. M. Chamberlain, L. Bodrossy, N. Stralis-Pavese, and J. C. Murrell.** 2008. Diversity of the active methanotrophic community in acidic peatlands as assessed by mRNA and SIP-PLFA analyses. Environ. Microbiol. **10:**446–459.
8. **Dalal, R. C., and D. E. Allen.** 2008. Greenhouse gas fluxes from natural ecosystems. Aust. J. Bot. **56:**369–407.
9. **Dedysh, S. N., C. Knief, and P. F. Dunfield.** 2005. *Methylocella* species are facultatively methanotrophic. J. Bacteriol. **187:**4665–4670.
10. **Dedysh, S. N., W. Liesack, V. N. Khmelenina, N. E. Suzina, Y. A. Trotsenko, J. D. Semrau, A. M. Bares, N. S. Panikov, and J. M. Tiedje.** 2000. *Methylocella palustris* gen. nov., sp nov., a new methane-oxidizing acidophilic bacterium from peat bags, representing a novel subtype of serine-pathway methanotrophs. Int. J. Syst. Evol. Microbiol. **50:**955–969.
11. **Degelmann, D. M., W. Borken, and S. Kolb.** 2009. Methane oxidation kinetics differ in European beech and Norway spruce soils. Eur. J. Soil Sci. **60:**499–506.
12. **Denman, K. L., G. Brasseur, A. Chidthaisong, P. Ciais, P. M. Cox, R. E. Dickinson, D. Hauglustaine, C. Heinze, E. Holland, D. Jacob, U. Lohmann, S. Ramachandran, P. L. da Silva Dias, S. C. Wofsy, and X. Zhang.** 2007. Couplings between changes in the climate system and biogeochemistry, p. 499–587. *In* S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, M. Tignor, and H. L. Miller (ed.), Climate change 2007: the physical science basis. Contribution of working group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom.
13. **Dunfield, P., and R. Knowles.** 1995. Kinetics of inhibition of methane oxidation by nitrate, nitrite, and ammonium in a humisol. Appl. Environ. Microbiol. **61:**3129–3135.
14. **Dunfield, P. F.** 2007. The soil methane sink, p. 152–170. *In* D. Reay, C. N. Hewitt, K. Smith, and J. Grace (ed.), Greenhouse gas sinks. CABI Publishing, Wallingfort, United Kingdom.
15. **Dunfield, P. F., V. N. Khmelenina, N. E. Suzina, Y. A. Trotsenko, and S. N. Dedysh.** 2003. *Methylocella silvestris* sp. nov., a novel methanotroph isolated from an acidic forest cambisol. Int. J. Syst. Evol. Microbiol. **53:**1231–1239.
16. **Dunfield, P. F., W. Liesack, T. Henckel, R. Knowles, and R. Conrad.** 1999. High-affinity methane oxidation by a soil enrichment culture containing a type II methanotroph. Appl. Environ. Microbiol. **65:**1009–1014.
17. **Dunfield, P. F., A. Yuryev, P. Senin, A. V. Smirnova, M. B. Stott, S. Hou, B. Ly, J. H. Saw, Z. Zhou, Y. Ren, J. Wang, B. W. Mountain, M. A. Crowe, T. M. Weatherby, P. L. E. Bodelier, W. Liesack, L. Feng, L. Wang, and M. Alam.** 2007. Methane oxidation by an extremely acidophilic bacterium of the phylum *Verrucomicrobia*. Nature **450:**879–882.
18. **Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution **39:**783–791.
19. **Heyer, J., V. F. Galchenko, and P. F. Dunfield.** 2002. Molecular phylogeny of type II methane-oxidizing bacteria isolated from various environments. Microbiology **148:**2831–2846.
20. **Holmes, A. J., N. J. Owens, and J. C. Murrell.** 1995. Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. Microbiology **141:**1947–1955.
21. **Holmes, A. J., P. Roslev, I. R. McDonald, N. Iversen, K. Henriksen, and J. C. Murrell.** 1999. Characterization of methanotrophic bacterial populations in soils showing atmospheric methane uptake. Appl. Environ. Microbiol. **65:**3312–3318.
22. **Hutchens, E., S. Radajewski, M. G. Dumont, I. R. McDonald, and J. C. Murrell.** 2004. Analysis of methanotrophic bacteria in Movile Cave by stable isotope probing. Environ. Microbiol. **6:**111–120.
23. **Islam, T., S. Jensen, L. J. Reigstad, O. Larsen, and N. K. Birkeland.** 2008. Methane oxidation at 55°C and pH 2 by a thermoacidophilic bacterium belonging to the *Verrucomicrobia* phylum. Proc. Natl. Acad. Sci. U. S. A. **105:**300–304.
24. **Jaatinen, K., C. Knief, P. F. Dunfield, K. Yrjälä, and H. Fritze.** 2004. Methanotrophic bacteria in boreal forest soil after fire. FEMS Microbiol. Ecol. **50:**195–202.
25. **Jones, D. T., W. R. Taylor, and J. M. Thornton.** 1992. The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. **8:**275–282.
26. **King, G. M., and K. Nanba.** 2008. Distribution of atmospheric methane oxidation and methanotrophic communities in Hawaiian volcanic deposits and soils. Microbes Environ. **23:**326–330.
27. **Klappenbach, J. A., P. R. Saxman, J. R. Cole, and T. M. Schmidt.** 2001. rrndb: the ribosomal RNA operon copy number database. Nucleic Acids Res. **29:**181–184.
28. **Knief, C., and P. F. Dunfield.** 2005. Response and adaptation of different methanotrophic bacteria to low methane mixing ratios. Environ. Microbiol. **7:**1307–1317.
29. **Knief, C., S. Kolb, P. L. Bodelier, A. Lipski, and P. F. Dunfield.** 2006. The active methanotrophic community in hydromorphic soils changes in response to changing methane concentration. Environ. Microbiol. **8:**321–333.
30. **Knief, C., A. Lipski, and P. F. Dunfield.** 2003. Diversity and activity of methanotrophic bacteria in different upland soils. Appl. Environ. Microbiol. **69:**6703–6714.
31. **Knief, C., S. Vanitchung, N. W. Harvey, R. Conrad, P. F. Dunfield, and A. Chidthaisong.** 2005. Diversity of methanotrophic bacteria in tropical upland soils under different land uses. Appl. Environ. Microbiol. **71:**3826–3831.
32. **Kolb, S.** 2009. The quest for atmospheric methane oxidizers in forest soils. Environ. Microbiol. Rep. **1:**336–346.
33. **Kolb, S., C. Knief, P. F. Dunfield, and R. Conrad.** 2005. Abundance and activity of uncultured methanotrophic bacteria involved in the consumption of atmospheric methane in two forest soils. Environ. Microbiol. **7:**1150–1161.
34. **Kolb, S., C. Knief, S. Stubner, and R. Conrad.** 2003. Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl. Environ. Microbiol. **69:**2423–2429.
35. **Lane, D. J.** 1991. 16S/23S rRNA sequencing, p. 115–175. *In* E. Stackebrandt and M. Goodfellow (ed.), Nucleic acid techniques in bacterial systematics. John Wiley and Sons Ltd., Chichester, United Kingdom.
36. **Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer.** 2004. ARB: a software environment for sequence data. Nucleic Acids Res. **32:**1363–1371.
37. **Maurer, D., S. Kolb, L. Haumeier, and W. Borken.** 2008. Inhibition of atmospheric methane oxidation by monoterpenes in Norway spruce and European beech soils. Soil Biol. Biochem. **40:**3014–3020.
38. **McDonald, I. R., L. Bodrossy, Y. Chen, and J. C. Murrell.** 2008. Molecular ecology techniques for the study of aerobic methanotrophs. Appl. Environ. Microbiol. **74:**1305–1315.
39. **Menyailo, O. V., W. R. Abraham, and R. Conrad.** 2010. Tree species affect atmospheric CH_4 oxidation without altering community composition of soil methanotrophs. Soil Biol. Biochem. **42:**101–107.
40. **Menyailo, O. V., and B. A. Hungate.** 2003. Interactive effects of tree species and soil moisture on methane consumption. Soil Biol. Biochem. **35:**625–628.
41. **Messing, J.** 1983. New M13 vectors for cloning. Methods Enzymol. **101:**20–78.
42. **Muyzer, G., E. C. Dewaal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S ribosomal RNA. Appl. Environ. Microbiol. **59:**695–700.
43. **Op den Camp, H. J. M., T. Islam, M. B. Stott, H. R. Harhangi, A. Hynes, S. Schouten, M. S. M. Jetten, N. K. Birkeland, A. Pol, and P. F. Dunfield.** 2009. Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. Environ. Microbiol. Rep. **1:**293–306.
44. **Pol, A., K. Heijmans, H. R. Harhangi, D. Tedesco, M. S. M. Jetten, and**

H. J. M. Op den Camp. 2007. Methanotrophy below pH 1 by a new *Verrucomicrobia* species. Nature **450:**874–878.
45. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
46. **Schloss, P. D., and J. Handelsman.** 2005. Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. Appl. Environ. Microbiol. **71:**1501–1506.
47. **Schloss, P. D., B. R. Larget, and J. Handelsman.** 2004. Integration of microbial ecology and statistics: a test to compare gene libraries. Appl. Environ. Microbiol. **70:**5485–5492.
48. **Smith, K. A., K. E. Dobbie, B. C. Ball, L. R. Bakken, B. K. Sitaula, S. Hansen, R. Brumme, W. Borken, S. Christensen, A. Prieme, D. Fowler, J. A. Macdonald, U. Skiba, L. Klemedtsson, A. Kasimir-Klemedtsson, A. Degorska, and P. Orlanski.** 2000. Oxidation of atmospheric methane in Northern European soils, comparison with other ecosystems, and uncertainties in the global terrestrial sink. Glob. Chang. Biol. **6:**791–803.
49. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol. **44:**846–849.
50. **Stolyar, S., A. M. Costello, T. L. Peeples, and M. E. Lidstrom.** 1999. Role of multiple gene copies in particulate methane monooxygenase activity in the methane-oxidizing bacterium *Methylococcus capsulatus* Bath. Microbiology **145:** 1235–1244.
51. **Stralis-Pavese, N., A. Sessitsch, A. Weilharter, T. Reichenauer, J. Riesing, J. Csontos, J. C. Murrell, and L. Bodrossy.** 2004. Optimization of diagnostic microarray for application in analysing landfill methanotroph communities under different plant covers. Environ. Microbiol. **6:**347–363.
52. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
53. **Trotsenko, Y. A., and J. C. Murrell.** 2008. Metabolic aspects of aerobic obligate methanotrophy. Adv. Appl. Microbiol. **63:**183–229.
54. **Wintzingerode, F. V., U. B. Goebel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:**213–229.
55. **Zaprasis, A., Y. J. Liu, S. J. Liu, H. L. Drake, and M. A. Horn.** 2010. Abundance of novel and diverse *tfdA*-like genes, encoding putative phenoxyalkanoic acid herbicide-degrading dioxygenases, in soil. Appl. Environ. Microbiol. **76:**119–128.