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Diversity and Activity of Methanotrophic Bacteria in Different Upland Soils

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Samples from diverse upland soils that oxidize atmospheric methane were characterized with regard to methane oxidation activity and the community composition of methanotrophic bacteria (MB). MB were identified on the basis of the detection and comparative sequence analysis of the *pmoA* gene, which encodes a subunit of particulate methane monooxygenase. MB commonly detected in soils were closely related to *Methylocaldum* spp., *Methylosinus* spp., *Methylocystis* spp., or the "forest sequence cluster" (USC α), which has previously been detected in upland soils and is related to *pmoA* sequences of type II MB (*Alphaproteobacteria*). As well, a novel group of sequences distantly related (<75% derived amino acid identity) to those of known type I MB (*Gammaproteobacteria*) was often detected. This novel "upland soil cluster γ" (USC γ) was significantly more likely to be detected in soils with pH values of greater than 6.0 than in more acidic soils. To identify active MB, four selected soils were incubated with $^{13}CH_4$ at low mixing ratios (<50 ppm of volume), and extracted methylated phospholipid fatty acids (PLFAs) were analyzed by gas chromatography-online combustion isotope ratio mass spectrometry. Incorporation of ^{13}C into PLFAs characteristic for methanotrophic *Gammaproteobacteria* was observed in all soils in which USC γ sequences were detected, suggesting that the bacteria possessing these sequences were active methanotrophs. A pattern of labeled PLFAs typical for methanotrophic *Alphaproteobacteria* was obtained for a sample in which only USC α sequences were detected. The data indicate that different MB are present and active in different soils that oxidize atmospheric methane.

Methane (CH_4) is present in the atmosphere at a mixing ratio of about 1.7 ppm of volume (ppmv). An estimated 30 Tg of CH_4 from the atmosphere $year^{-1}$ is oxidized by aerobic methanotrophic bacteria (MB) in upland soils, accounting for about 6% of the global atmospheric CH_4 sink (21, 31). Bender and Conrad (2) suggested that MB active in upland soils are specialized oligotrophs adapted to the trace level of atmospheric CH_4 and possess a methane monooxygenase (MMO) with a higher substrate affinity than that of cultivated MB. It was later demonstrated that the application of single-reactant Michaelis-Menten kinetics to MMO is not always appropriate and that the apparent affinity for CH_4 varies depending on the cultivation conditions (13). Nevertheless, recent studies indicate that MB in at least some soils that oxidize atmospheric CH_4 are indeed taxonomically distinct from known MB (28).

The 13 recognized genera of MB are divided into two groups, type I (further divided into types I and X) and type II. These differ in phylogenetic affiliation (*Gammaproteobacteria* versus *Alphaproteobacteria*) and in diverse biochemical characteristics (21). Identification of MB in soils is often performed by the cultivation-independent detection of a fragment of *pmoA*, a gene encoding the active-site subunit of particulate MMO (22, 26, 30, 35, 38). This marker gene is present in all known MB, with the exception of *Methylocella palustris* (12) and *Methylocella silvestris* (14). Sequence-based *pmoA* phylogeny correlates well with 16S rRNA-based phylogeny, so *pmoA* sequences can be assigned to specific genera or even species of MB (24, 28, 35). The *pmoA* gene is therefore an excellent functional gene marker and has been widely used to characterize methanotrophic communities in soils, including upland soils that consume atmospheric methane (4, 18, 23, 28, 32, 42, 45). In some of these soils, a novel sequence cluster usually called the "forest sequence cluster" (here USC α) has been detected (4, 23, 28, 32). The most closely related *pmoA* sequence from a pure culture is that of *Methylocapsa acidiphila*, a type II MB isolated from acidic peat (11). Besides this novel sequence cluster, *pmoA* sequences related to the genera *Methylocystis*, *Methylosinus*, *Methylomonas*, *Methylobacter*, *Methylomicrobium*, *Methylococcus*, and *Methylocaldum* have been detected in upland soils (4, 28, 32, 42, 45).

Detection of a particular *pmoA* gene in a soil does not necessarily imply that the respective methanotroph is physiologically active in this soil. Thus, it remains unclear whether all of the genera of MB detected in upland soils are involved in the process of atmospheric methane oxidation. Holmes et al. (28) combined *pmoA* analysis of soils with ^{14}C labeling of phospholipid fatty acids (PLFAs), which are useful biomarkers with which to distinguish different groups of MB (6, 19). Soils were incubated with $^{14}CH_4$, and the resulting ^{14}C-labeled PLFA profiles were similar but not identical to the PLFA profiles of type II MB (*Alphaproteobacteria*). Only *pmoA* sequences of USC α were detected in these soil samples. On the basis of these combined results, the existence of a novel group of methanotrophic *Alphaproteobacteria* involved in atmospheric methane oxidation was postulated. Later work with $^{14}CH_4$ or $^{13}CH_4$ labeling of PLFAs has supported this hypothesis (8, 44). Soil pH values were reported in two of these studies, and all were acidic (pH ≤5.2) (28, 44). The close relationship of the novel *pmoA* sequences to that of the aci-

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TABLE 1. Descriptions of sampling sites, soil pHs, and kinetic values of methane oxidation[a]

Location or time and soil sample	Position	Soil type, German classification (corresponding FAO[c] soil group)	Land use or plant cover	pH	$V_{max(app)}$	a^0_s	$K_{m(app)}$
Eiterfeld							
E 20	50°45.406′N, 9°49.721′E	Parabraunerde (Luvisol)	Farmland (wintergrain)	7.6	0.00		
E 33a	50°45.252′N, 9°48.678′E	Pseudogley Parabraunerde (Gleyic Luvisol)	Deciduous forest (*Fagus sylvatica*, *Quercus* sp.)	6.6	0.94	0.63	37
E 33b	50°45.243′N, 9°48.675′E	Pseudogley Parabraunerde (Gleyic Luvisol)	Deciduous forest (*Fagus sylvatica*, *Quercus* sp.)	5.1	0.08	0.31	6
E L4	50°44.991′N, 9°48.371′E	Pararendzina (Leptosol)	Meadow, scattered with *Pinus sylvestris*, sampling point from meadow	5.7	0.25	0.50	12
E L4P	50°44.990′N, 9°48.374′E	Pararendzina (Leptosol)	Meadow, scattered with *Pinus sylvestris*, sampling point under *Pinus sylvestris*	5.9	0.13	0.37	9
E L5	50°45.034′N, 9°48.496′E	Pseudogley Parabraunerde (Gleyic Luvisol)	Deciduous forest (*Fagus sylvatica*, *Quercus* sp., *Prunus* sp.)	5.2	0.11	0.10	29
E L6	50°45.002′N, 9°48.400′E	Pararendzina (Leptosol)	Meadow	6.2	0.45	0.41	26
E W7	50°43.654′N, 9°43.654′E	Rendzina (Leptosol)	Meadow	7.2	0.01		
E 5FB	50°43.461′N, 9°47.118′E	Rendzina (Leptosol)	Mixed forest (*Fagus sylvatica*, *Quercus* sp., scattered *Pinus sylvestris*)	7.5	3.11	1.36	56
E 5FL	50°43.463′N, 9°47.081′E	Rendzina (Leptosol)	Mixed forest (*Pinus sylvestris*, *Larix decidua*, scattered *Fagus sylvatica*)	6.2	0.48	0.10	114
E 26F	50°43.023′N, 9°48.501′E	Parabraunerde (Luvisol)	Deciduous forest (*Fagus sylvatica*, *Quercus* sp.)	4.9	0.07	0.08	21
E 26W	50°43.010′N, 9°48.507′E	Parabraunerde (Luvisol)	Pasture	5.9	0.24	0.08	78
E 26A	50°43.008′N, 9°48.504′E	Parabraunerde (Luvisol)	Farmland (wintergrain)	6.6	0.04	0.06	17
E 56A	50°42.971′N, 9°45.506′E	Pseudogley (Stagnic Gleysol)	Farmland (rape)	6.9	0.01	0.03	12
E 56W	50°42.971′N, 9°45.506′E	Pseudogley (Stagnic Gleysol)	Pasture	5.6	0.11	0.32	8
E 56F	50°42.960′N, 9°45.423′E	Pseudogley (Stagnic Gleysol)	Mixed forest (*Pinus sylvestris*, *Larix decidua*, *Fagus sylvatica*, *Quercus rubra*, *Populus tremula*)	4.6	0.16	0.45	9
Gerold							
GE BWN	47°28.950′N, 11°11.967′E	Not determined	Meadow	5.8	0.51	0.67	18
GE BF	47°28.950′N, 11°11.967′E	Not determined	Mixed forest (*Fagus sylvatica*, *Picea abies*)	6.8	0.80	0.73	27
Welschnofen/ Völser Aicha							
WO DFH	46°25.342′N, 11°36.030′E	Not determined	Coniferous forest (*Picea abies*, *Pinus cembra*, *Larix decidua*)	5.6	1.55	1.74	22
VA DFT	46°29.204′N, 11°29.324′E	Not determined	Mixed forest (*Fagus sylvatica*, *Picea abies*)	4.4	1.11	6.41	4
VA DWT	6°29.294′N, 11°29.318′E	Not determined	Meadow	6.8	0.77	0.64	30
Marburg, MF	51°00.000′N, 9°50.625′E	Braunerde (Cambisol)	Mixed forest (*Fagus sylvatica*, *Quercus robur*)	4.0	1.26	5.08	6
Bad Bentheim/ Denekamp							
BB NH	51°21.505′N, 7°04.285′E	Podsole (Podzol)	Heathland (*Calluna vulgaris*)	4.6	0.08	0.24	8
BB NNW	51°21.383′N, 7°04.267′E	Podsole (Podzol)	Coniferous forest (*Pinus sylvestris*)	4.3	0.06	0.32	4
BB WNW[b]	51°21.104′N, 7°06.169′E	Podsole (Podzol)	Coniferous forest (*Pinus sylvestris*)	3.9	0.01	0.16	2
BB WAS	51°21.004′N, 7°06.190′E	Podsole (Podzol)	Farmland (closed for 0.5 yr)	5.6	0.25	0.11	58
Göttingen							
G 15F	51°33.974′N, 10°0.657′E	Pseudogley Parabraunerde (Gleyic Luvisol)	Deciduous forest (*Fagus sylvatica*, *scattered Tilia platiphyllos*, *Acer pseudoplatanus*)	7.7	0.95	0.39	60
G 15W	51°33.971′N, 10°0.645′E	Pseudogley Parabraunerde (Gleyic Luvisol)	Meadow	7.3	0.39	0.05	196
G 15A[b]	51°33.964′N, 10°0.655′E	Pseudogley Parabraunerde (Gleyic, Luvisol)	Farmland (wintergrain)	7.8	0.05	0.03	38
G 13FH	51°31.096′N, 10°2.171′E	Rendzina (Leptosol)	Deciduous forest (*Fagus sylvatica*, *Fraxinus excelsior*, *Acer platanoides*)	7.1	0.93	0.23	100
G 44W	51°30.411′N, 10°1.154′E	Rendzina (Leptosol)	Meadow	7.8	1.22	1.51	20
G 44F	51°30.423′N, 10°1.167′E	Rendzina (Leptosol)	Deciduous forest (*Fagus sylvatica*, *Fraxinus excelsior*)	8.0	2.39	1.00	58

Würzburg							
W B5FH	49°49.041′N, 9°54.955′E	Podsolige Braunerde (Cambisol)	Mixed forest (*Fagus sylvatica*, *Carpinus betulus*, *Pinus sylvestris*, *Larix decidua*)	6.3	0.44	0.33	33
W B5FP	49°48.960′N, 9°54.804′E	Podsolige Braunerde (Cambisol)	Mixed forest (*Acer pseudoplatanus*, *Fagus sylvatica*, *Quercus robur*, *Pinus sylvestris*)	6.9	0.49	0.20	60
W Re4F	49°51.540′N, 9°54.521′E	Protorendzina (Leptosol)	Deciduous forest (*Carpinus betulus*, *Prunus* sp., *Quercus robur*, *Acer campestris*)	6.1	0.29	0.15	46
W Re1F	49°52.553′N, 9°52.355′E	Protorendzina (Leptosol)	Mixed forest (*Pinus sylvestris*, *Quercus petraea*, *Fagus sylvatica*, *Acer campestre*)	7.6	0.37	0.15	58
W Re3W	49°52.234′N, 9°51.684′E	Mullartige Rendzina (Leptosol)	Meadow	7.8	0.74	0.34	53
July 2002							
E 5FB	50°43.461′N, 9°47.118′E	Rendzina (Leptosol)	Mixed forest (*Fagus sylvatica*, *Quercus* sp., scattered *Pinus sylvestris*)	6.5	1.52	0.21	177
MF	51°00.000′N, 9°50.625′E	Braunerde (Cambisol)	Mixed forest (*Fagus sylvatica*, *Quercus robur*)	4.3	2.62	1.58	40
G 15F	51°33.974′N, 10°0.657′E	Pseudogley Parabraunerde (Gleyic Luvisol)	Deciduous forest (*Fagus sylvatica*, scattered *Tilia platiphyllas*, *Acer pseudoplatanus*)	7.9	0.78	1.53	13
G 13FH	51°31.096′N, 10°2.171′E	Rendzina (Leptosol)	Deciduous forest (*Fagus sylvatica*, *Fraxinus excelsior*, *Acer platanoides*)	6.8	0.80		
G 44F	51°30.423′N, 10°1.167′E	Rendzina (Leptosol)	Deciduous forest (*Fagus sylvatica*, *Fraxinus excelsior*)	7.9	1.80	1.02	43
G 44W	51°30.411′N, 10°1.154′E	Rendzina (Leptosol)	Meadow	8.0	1.38		

[a] Kinetic values are the maximum zero-order methane uptake rate ($V_{max(app)}$ [nanomoles of CH_4 per gram of dry weight per hour]), the apparent Michaelis-Menten kinetic constant ($K_{m(app)}$ [ppmv of CH_4]), which was calculated from $V_{max(app)}/a^0_s$, and the specific affinity (a^0_s [milliliters of gas per gram of dry weight per hour]). a^0_s is a first-order rate constant. The unit milliliters refers not to milliliters of CH_4 but rather to standardization to a 1-ml total gas volume. To calculate rates of atmospheric methane uptake at 25°C multiply a^0_s by the mixing ratio of methane in the atmosphere (1.7 ppmv of CH_4 = 0.071 nmol of CH_4 ml^{-1}).

[b] Sample was not analyzed by molecular methods.

[c] FAO, Food and Agriculture Organization of the United Nations.

dophile *M. acidiphila* may indicate that the putative MB possessing these sequences are specialized acidophiles.

The influence of various physicochemical soil parameters (temperature, water content, pH, ammonium content, land use, fertilization, and texture) on methane oxidation activity in upland soils has been examined in several studies (36). The influence of these parameters on the composition of the methanotrophic community has only rarely been studied, e.g., the vertical distribution of MB in a soil (23) or the effects of land use (42). The aim of our study was to characterize MB communities and methane oxidation activity in diverse upland soils varying in pH, soil type, land use, and plant cover. To characterize the community of physiologically active MB, selected soil samples were incubated with $^{13}CH_4$, followed by extraction and analysis of PLFAs.

MATERIALS AND METHODS

Sampling sites and soil characteristics. Sampling sites were located near Eiterfeld, Bad Bentheim, Gerold, Marburg, Würzburg, and Göttingen (Germany), near Welschnofen and Völser Aicha (Italy), and near Denekamp (The Netherlands) (Table 1). Soil samples were taken in May to July 2001 and in April 2002 from the upper mineral horizon (generally 5 to 20 cm from the soil surface). The soil types were determined by reference to soil maps (1:25,000). Further characterization of the soil samples included measurements of pH in water and ammonium concentration (33). On the basis of the initial results, sites E 5FB, G 15F, G 13FH, G 44F, G 44W, and MF were selected for further analyses, and fresh samples of these soils were taken in July 2002.

Methane oxidation. Ten-gram amounts of sieved (<3-mm mesh) soil were incubated at 25°C in 120-ml serum vials closed with butyl rubber septa. Triplicates of each sample were incubated under atmospheric CH_4 or under elevated CH_4 mixing ratios (100 to 400 ppmv). The decrease in CH_4 in the headspace was measured with an SRI 8610C gas chromatograph (SRI Instruments, Torrance, Calif.) equipped with a flame ionization detector (GC-FID) (detector temperature, 140°C; Porapak Q column, 6 feet long, 1/8 in. in diameter, 80/100 mesh [Supelco, Taufkirchen, Germany]; oven temperature, 100°C). A linear decrease in CH_4 versus time was always observed in the elevated-CH_4 vials and was used to estimate maximum apparent CH_4 oxidation rates [$V_{max(app)}$]. Incubation under atmospheric CH_4 mixing ratios resulted in an exponential decrease in CH_4, from which the specific affinity a^0_s (first-order uptake rate constant) was calculated by using the least-squares iterative fitting procedure of Origin 6.1 (Microcal Software, Inc., Northampton, Maine). Incubation times varied depending on the activity of the sample and included 3 to 12 measurement points (elevated CH_4) or 4 to 10 measurement points (atmospheric CH_4).

Methane oxidation kinetics were determined more intensely in the fresh samples collected for the $^{13}CH_4$-labeling experiment. Vials (35 ml) were filled with 5 ml of soil slurry (2 volumes of distilled water per g of soil), injected with CH_4 mixing ratios of 2 to 150 ppmv (duplicates at each level), and incubated on a rotary shaker at 180 rpm and 25°C. CH_4 oxidation rates were estimated by linear regression (maximum decline in CH_4 of 33%, maximum incubation time of 34 h) and plotted against the CH_4 concentration at the time midpoint. $V_{max(app)}$ and the apparent half-saturation constant [$K_{m(app)}$] were estimated by least-squares fitting to a Michaelis-Menten hyperbolic model.

DNA extraction and PCR amplification. DNA was extracted from 0.5 g of soil (stored at −20°C immediately after sampling) with a Fast DNA SPIN Kit (Bio 101, La Jolla, Calif.). To increase DNA recovery, the final elution of the DNA was performed twice with 100 μl of DNase-free water. Additional purification was achieved with polyvinylpolypyrrolidone as described by Henckel et al. (23), again with twofold elution of the DNA with TE buffer (10 mM Tris base, 1 mM EDTA, pH 8). The DNA was finally purified and concentrated with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany).

A partial fragment of *pmoA* was amplified with primers A189f and A682b (38) and primers A189f and mb661 (10). For denaturing gradient gel electrophoresis (DGGE), a GC clamp was attached to the 5′ end of the A189f primer (22). A touchdown PCR program (22) with annealing temperatures decreasing from 62 to 55°C was used with 30 cycles when primers A189f and A682b were used and 35 cycles when primers A189f and mb661 were used. A fragment of the *mmoX* gene encoding the active-site subunit of soluble MMO was amplified with primers described by Auman et al. (1). The PCR program consisted of an initial denaturation step of 5 min at 94°C, followed by 35 cycles of 94°C for 1 min, 55°C

for 1.5 min, and 72°C for 1 min, with a final extension step of 72°C for 7 min. Amplification of a gene fragment of the 16S rRNA was performed with primers UNI 533f and UNI 907r (47) as described previously (22). All PCR mixtures contained each primer at 0.5 μM, 1× Premix F (Epicentre Technologies, Madison, Wis.), 1 U of *Taq* DNA polymerase (Qbiogene, Heidelberg, Germany), and 1 μl of template DNA and were run on either a GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems, Weiterstadt, Germany) or a Primus 96 Cycler (MWG Biotech, Ebersberg, Germany).

DGGE. For each sample, separation of the mixed *pmoA* products by DGGE was performed on PCR products of both primers A189f and A682b and primers A189f and mb661. DGGE was performed as described previously (22), in a gradient of 35 to 80% denaturant at 61°C and 180 V for 5 h. Visible bands were excised and reamplified. PCR products of bands that migrated closely in DGGE gels were checked for purity in a second DGGE gel. Although reamplification of bands usually gave a single *pmoA* product to be sequenced, occasionally nonspecific products were also evident. Three procedures were used to remove these nonspecific products: (i) primer mb661 or A650 (4), instead of A682b, was used for reamplification of excised bands; (ii) the complete PCR product was loaded onto an agarose gel, and the band with the expected size of *pmoA* was excised and purified with a QIAquick Gel Extraction Kit (Qiagen); or (iii) PCR products were cloned with a TOPO TA cloning kit (Invitrogen, Karlsruhe, Germany). The *pmoA* products obtained from positive clones were verified by comparing their migration in DGGE to that of the original excised *pmoA* gene fragment.

Sequencing and phylogenetic analysis. PCR products from excised DGGE bands were purified with a QIAquick PCR Purification Kit (Qiagen) and sequenced on an ABI 377 DNA sequencer with BigDye terminator chemistry as specified by the manufacturer (Perkin-Elmer Applied Biosystems). Phylogenetic tree reconstructions based on deduced amino acid sequences of partial *pmoA* and *amoA* sequences were performed with the ARB software package (46) and the PHYLIP software package, version 3.6a2.1 (17). Original tree construction included sequences of all DGGE bands, all available *pmoA* sequences in the GenBank database (April 2003), and selected public-domain *amoA* sequences. A selection was then made of 28 representative sequences from this study plus 38 public-domain sequences.

Labeling experiments with [^{13}C]methane. Incubation of 25-g amounts of sieved soil under 50 ppmv of labeled $^{13}CH_4$ (99%; Cambridge Isotope Laboratories, Mass.) or unlabeled CH_4 in air was performed in 1,000-ml screw-cap flasks sealed gas tight with silicone septa. Incubation was performed at 25°C for 4 weeks. Methane was measured at 1- to 5-day intervals (depending on the activity of the sample) by GC-FID and added again each time the mixing ratio fell below 10 ppmv. Control labeling experiments were also done with the MB strains *Methylosinus trichosporium* KS24b (24) and *Methylocaldum* sp. strain E10a. The latter was isolated from site E 20 by methods described previously (24). Its 16S rRNA sequence is 97.8% identical to that of *Methylocaldum tepidum* LK6. Cultures were grown in liquid mineral salts medium A under an atmosphere containing 20% (vol/vol) CH_4 (25).

Care was taken to control for secondary labeling effects of $^{13}CO_2$ in soil incubations. Tubes containing 5 ml of 5 M NaOH solution were inserted into flasks to fix emerging $^{13}CO_2$. After 2 weeks of incubation, the $^{13}C/^{12}C$ isotope ratio of CO_2 in the headspace of the flasks was measured with a Thermo Finnigan MAT Delta Plus isotope mass spectrometer (Thermoquest, Bremen, Germany) coupled to a Hewlett-Packard (Waldbronn, Germany) 6890 gas chromatograph (detector temperature, 150°C; Pora Plot Q column, 27.5 m by 0.32 mm [diameter] [Chrompack, Frankfurt, Germany]; oven temperature, 25°C) (GC-IRMS). This measurement detected a slight increase in labeled $^{13}CO_2$ in the headspace of samples incubated with $^{13}CH_4$ (increase in label in CO_2, <1 atom%). Therefore, the flasks were flushed well with air and the NaOH solution was replaced at this time. As a further control of secondary labeling effects, soil samples MF and G 44F were incubated with $^{13}CO_2$ instead of $^{13}CH_4$. $^{13}CO_2$ was released from a solution of $NaH^{13}CO_3$ (99.9%; Cambridge Isotope Laboratories) and added via syringe to the incubation flasks. The final mixing ratio of 225 ppmv of $^{13}CO_2$ was verified by measurement with a GC-8A GC-FID (Shimadzu, Kyoto, Japan) (detector temperature, 350°C; 50/100 mesh Porapak QS column, 2 m long by 1/8 in. in diameter [Alltech, Unterhaching, Germany]; oven temperature, 40°C) equipped with a methanizer (NiCr-Ni catalyst column, 20 cm long by 1/8 in. in diameter [Chrompack]). The $^{13}CO_2$ added was equal to the total amount of $^{13}CH_4$ oxidized in the soil sample with the highest methane oxidation activity.

PLFA analysis. Lipids were extracted from 10 g of soil by a modified Bligh and Dyer method and fractionated on silica columns (CUSIL15Z; ICT, Bad Homburg, Germany). PLFAs were subjected to mild alkaline methanolysis as described previously (34). Cells from pure cultures were harvested by centrifugation (10,000 × *g*, 20 min) and washed twice with 0.9% NaCl solution before PLFA extraction.

Separation, identification, and quantification of fatty acid methyl esters (FAMEs) were performed by gas chromatography mass spectrometry (GC-MS) as described previously (37). The positions of double bonds and cyclopropyl groups were determined by analysis of dimethyl disulfide adducts (39). Carbon isotope ratios of the individual FAMEs were determined with the GC-IRMS system described above. The GC-IRMS apparatus was equipped with the same nonpolar column as was the GC-MS apparatus (5% phenyl methyl silicone capillary column, 30 m by 0.25 mm [diameter] [Hewlett-Packard]), and the same temperature program was used to separate FAMEs (0.5 min at 120°C, increase of 5°C/min to 240°C and finally 240°C for 2 min) in order to obtain similar PLFA profiles by both analyses. The injector temperature of the GC-MS was set to 250°C, and a split ratio of 29.2 was used, while the injector temperature of the GC-IRMS system was set to 300°C and a splitless injection mode was used. To calculate isotope ratios ($\delta^{13}C$) for the PLFAs, $\delta^{13}C$ values of the FAMEs were corrected with a mass balance for the carbon atom of the methyl group that was added during methanolysis (43). For each PLFA, the incorporation of ^{13}C (*I*, expressed as micrograms of ^{13}C per gram of total PLFAs) was calculated as follows: $I = (F_l - F_u) \times (A_x)$, where A_x is the peak area of $PLFA_x$ divided by the sum of the peak areas of all of the PLFAs. *F* is the fraction of ^{13}C in $PLFA_x$ of samples incubated with ^{13}C (F_l) or samples incubated with ^{12}C (F_u) and was calculated as follows: $F = {}^{13}C/({}^{13}C + {}^{12}C) = R/(R + 1)$. The carbon isotope ratio (*R*) was derived from the measured $\delta^{13}C$ values as follows: $R = (\delta^{13}C/1{,}000 + 1) \times R_{VPDB}$, with $R_{VPDB} = 0.0112372$.

Nucleotide sequence accession numbers. Representative *pmoA* nucleotide sequences obtained during this study have been deposited in the GenBank, EMBL, and DDBJ nucleotide sequence databases under accession numbers AJ579657 to AJ579676.

RESULTS

Characterization of sampling sites. Soil samples were taken from seven regions in Central Europe incorporating different soil types, land uses, and plant covers (Table 1). Care was taken to select soil types of various pHs to test the hypothesis that USC α represents a specialized acidophile. Soil pH values ranged from 3.9 to 8.0.

No soil showed net CH_4 production, although a few had no measurable CH_4 uptake. The $V_{max(app)}$ values for methane oxidation at 25°C ranged from <0.01 to 3.11 nmol of CH_4 g of dry weight^{-1} h^{-1}. Specific affinity (a^0_s) reached values greater than 1 ml g of dry weight^{-1} h^{-1} in four forest soils and meadow soil sample G 44W. $K_{m(app)}$ values calculated from the data in Table 1 ranged from a CH_4 mixing ratio in air of 2 ppmv (BB WNW) to 196 ppmv (G 15W) (or 3 to 271 nM dissolved CH_4). A correlation between $V_{max(app)}$ and a^0_s was not observed, nor was there any significant correlation of these kinetic values with soil pH or ammonium or ammonia concentrations (data not shown).

Characterization of the MB community. The community of MB was characterized in 35 samples by cultivation-independent retrieval of partial *pmoA* genes, followed by comparative sequence analysis of derived amino acid sequences (PmoA). PCR amplification of *pmoA* from all samples was successful, with the exception of GEBWN, GEBF, and BB NH. Some of the sequences retrieved with primers A189f and A682b were closely related to the *amoA* gene (encoding a subunit of ammonia monooxygenase) of ammonia-oxidizing *Betaproteobacteria* (Fig. 1 and Table 2). This was expected, as primers A189f and A682b amplify both *pmoA* and *amoA* gene fragments, while A189f and mb661 are specific for *pmoA* (10, 27).

PmoA sequences closely related to those of the genera *Methylocaldum*, *Methylosinus*, and *Methylocystis* were detected in 70% of the soil samples analyzed (Table 2). *Methylocaldum*-

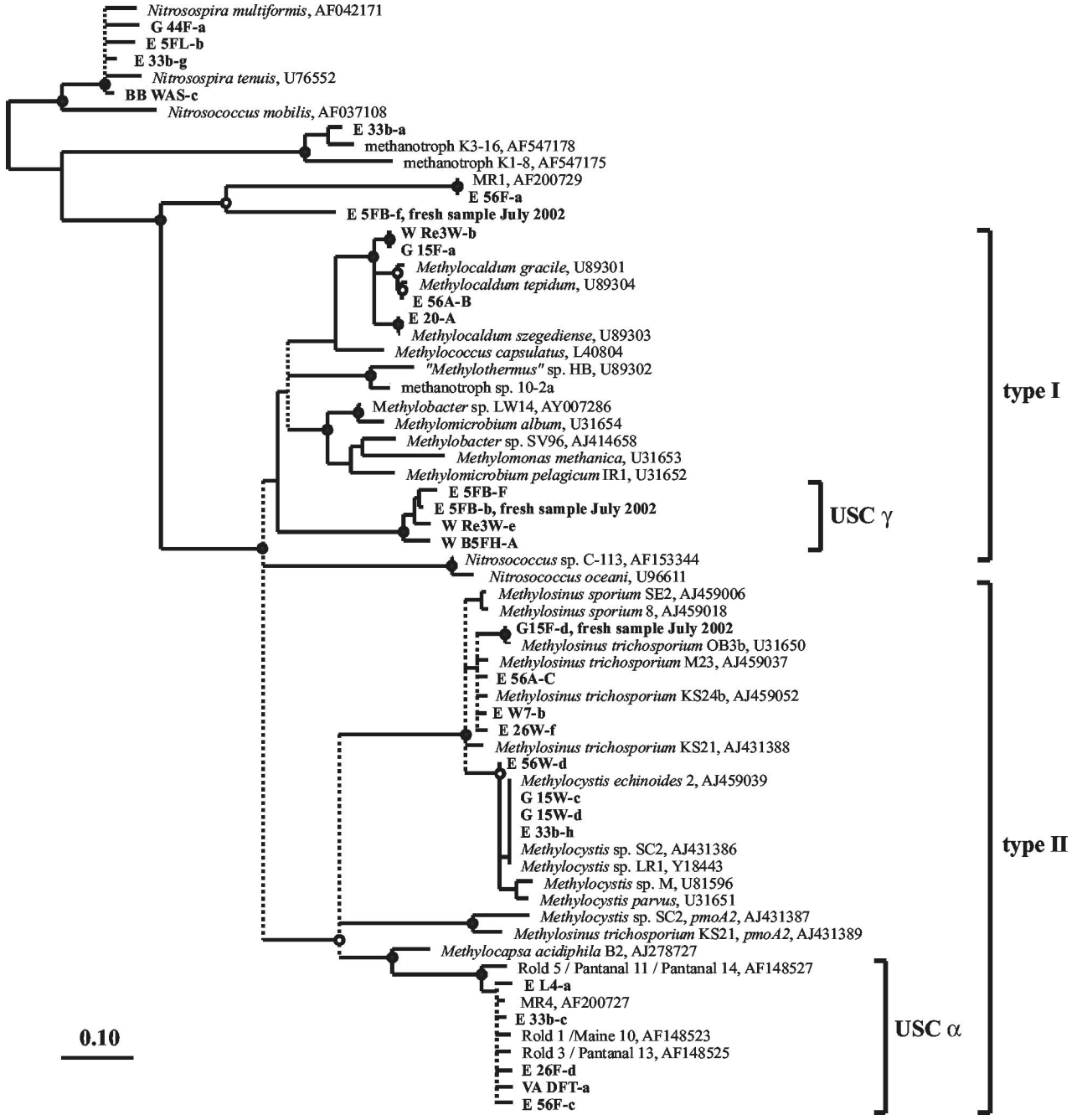

FIG. 1. Consensus tree showing the relationship of the amino acid sequences derived from partial *pmoA* sequences retrieved from soils to public-domain PmoA and AmoA sequences. The base tree was constructed on the basis of 141 amino acid residues with a neighbor-joining algorithm with a Kimura correction. Multifurcations indicate branch points where the topologies of distance matrix-based neighbor-joining trees with Kimura or PAM correction, of a maximum-likelihood tree, and of a parsimony tree differed. These are indicated by dashed lines. Bootstrap values were calculated with the PHYLIP software package on the basis of 1,000 data resamplings. Bootstrap values of ≥90% are indicated by black circles, and values of ≥80% are indicated by white circles. The cluster of AmoA sequences (top) was set as an outgroup. The bar represents 0.10 change per position.

like sequences were in three main groups: identical to those of *Methylocaldum szegediense*, closely related to *Methylocaldum tepidum* (>99% identity), and belonging to a novel branch with 94 to 95% identity to known *Methylocaldum* strains. The last are represented by sequences G 15F-a and W Re3W-b (Fig. 1). All *Methylosinus*-like PmoA sequences fell clearly within the *M. trichosporium* cluster. *Methylocystis*-like sequences clustered into a group represented by many sequences in the GenBank database, including *Methylocystis* sp. strain SC2 and *Methylocystis* sp. strain LR1 (Fig. 1).

Many soil samples also contained sequences less closely related to those of cultivated MB species. USC α (Table 2 and Fig. 1), which has previously been detected in upland soils that consume atmospheric methane, was very common. Another

TABLE 2. Methanotrophic community of different soil samples, based on detection of the *pmoA* gene[a]

Sample	pH	PmoA					PmoA or AmoA (unknown)[b]	AmoA (ammonia oxidizer)
		Methylocaldum	*Methylosinus*	*Methylocystis*	USC α	USC γ		
E 20	7.6	+	+					+
E 33a	6.6		+	+	+			+
E 33b	5.1		+	+	+		A	+
E L4	5.7		+		+			
E L4P	5.9		+		+			+
E L5	5.2		+		+		A	
E L6	6.2		+		+	+		
E W7	7.2	+	+	+	+			+
E 5FB	7.5		+			+		
E 5FL	6.2		+			+		+
E 26F	4.9		+		+			
E 26W	5.9		+	+	+			+
E 26A	6.6		+				A	+
E 56A	6.9	+	+				A	+
E 56W	5.6		+	+	+			
E 56F	4.6		+	+	+		B	
GE BWN	5.8							
GE BF	6.8							
WO DFH	5.6				+			
VA DFT	4.4				+			
VA DWT	6.8			+		+		
MF	4.0				+			
BB NH	4.6							
BB NNW	4.3				+			
BB WAS	5.6							+
G 15F	7.7	+		+	+			
G 15W	7.3			+				
G 13FH	7.1				+	+		
G 44W	7.8	+						
G 44F	8.0			+				+
W B5FH	6.3				+	+		
W B5FP	6.9	+			+			
W Re4F	6.1	+		+	+			
W Re1F	7.6	+		+		+		
W Re3W	7.8	+				+		
Samples from July 2002								
E 5FB	6.5				+	+	C	
MF	4.3				+			
G 15F	7.9		+		+	+		
G 13FH	6.8				+	+	B,C	+
G 44F	7.9		+	+	+	+		
G 44W	8.0		+		+	+		

[a] See Table 1 for site descriptions.
[b] A, B, and C indicate the affiliations of the detected sequences with different unknown clusters.

cluster of sequences (USC γ) was distantly related to PmoA sequences of methanotrophic *Gammaproteobacteria* (Fig. 1). The highest identity values of USC γ amino acid sequences to those of cultivated MB were only 72 to 75%, to *Methylocaldum* strains, *Methylococcus capsulatus*, and *Methylobacter* sp. strain LW14. In addition, soil samples E 33b, E L5, E 56A, and E 26A yielded PmoA sequences closely related to those of methanotrophic isolates K3-16 and K1-8, which were recently described by Pacheco-Oliver et al. (41). Two further sequence types may represent either AmoA or PmoA: (i) sequences from E 56F and G 13FH (collected in 2002) that were related to MR1 (23) and (ii) sequences represented by E 5FB-f, which were detected in soil samples E 5FB and G 13FH, which were collected in 2002 (Table 2).

A fragment of the *mmoX* gene was not detected in any sample. Either MB possessing soluble MMO are uncommon in upland soils, or the PCR systems used to detect *mmoX* are not as sensitive as those used for *pmoA*. Thus, no marker genes for methanotrophs were detected in the GEBWN, GEBF, and BB NH soil samples, although these soil samples exhibited methane oxidation activity (Table 1). Severe inhibition of the PCR assays of these three samples by coextracted inhibitory substances in the DNA extracts was unlikely, since PCR assays for the amplification of a partial 16S rRNA gene fragment were not inhibited (data not shown).

Relationship between soil pH and MB community composition. To gain insight into the distribution of MB in upland soils in relation to soil characteristics, the MB community composition of the different soil samples was analyzed in comparison to methane oxidation activity, land use, ammonium and am-

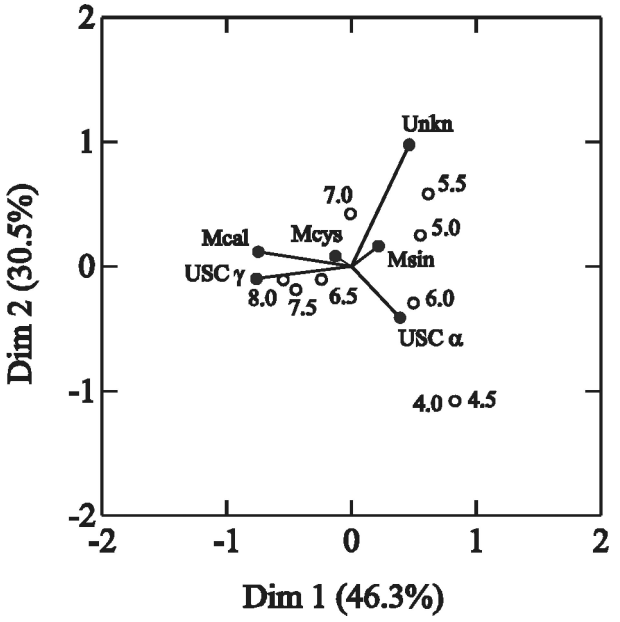

FIG. 2. Correspondence plot showing the relationship between soil pH values and the detection of the different *pmoA* sequences in 36 soil samples. The first two dimensions explain 76.8% of the variance among samples. The pH values were classified into nine groups and are represented by the empty circles in the plot. The different taxa are represented by filled circles and are indicated as follows: Mcal, *Methylocaldum* spp.; Mcys, *Methylocystis* spp.; Msin, *Methylosinus* spp.; Unkn, sequences of the unknown clusters that may represent either PmoA or (Dim1 and Dim2) AmoA.

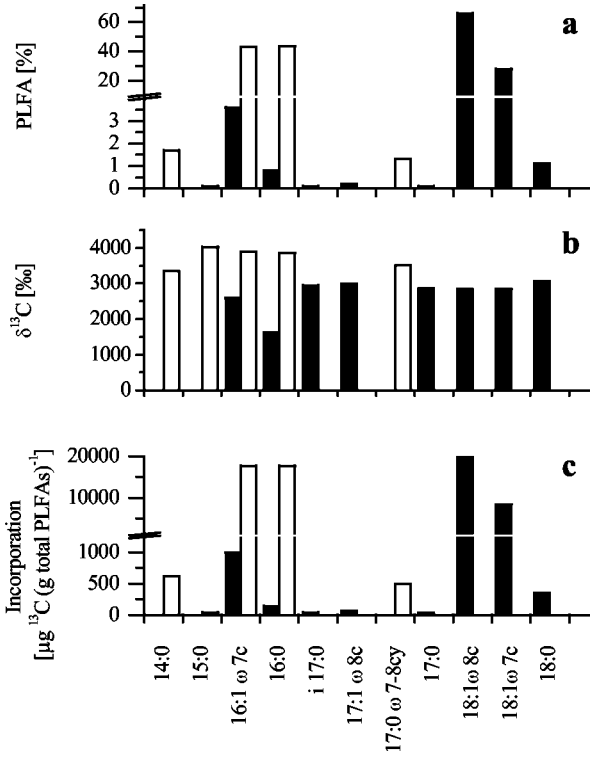

FIG. 3. PLFA profile and ^{13}C-labeling pattern of the PLFAs of *M. trichosporium* KS24b (filled bars) and *Methylocaldum* sp. strain E10a (open bars). The relative amount (a) and $\delta^{13}C$ value (b) of each PLFA were used to calculate ^{13}C incorporation into each PLFA relative to the total amount of PLFAs (c).

monia concentrations, and pH values by correspondence analyses with SYSTAT version 10.2 (SPSS Inc., Richmond, Calif.). Only pH showed a clear and significant influence on MB distribution, and only analyses for this factor are presented below.

The 36 soil samples from which *pmoA* products were retrieved (samples GE BWN, GE BF, BB NH, BB WNW, and G 15A excluded) and atmospheric methane uptake was detectable (samples E 20 and E W7 excluded) were grouped to the closest 0.5 pH unit (i.e., 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, and 8) in order to improve the degrees of freedom in the correspondence analyses, and the correspondence of pH levels to the species groups in Table 2 was calculated (Fig. 2). The occurrence of *Methylocaldum* spp. and USC γ strongly influenced the separation of samples with different pH values, such that the higher-pH soils corresponded well to the presence of these clusters. The significance of this trend in USC γ was confirmed with a contingency analysis. The null hypothesis that the USC γ sequences were equally distributed in high-pH versus low-pH soils (with the median soil pH of 6 chosen as the separation point between high and low) was rejected (Fisher's exact test, $P \leq 0.001$), indicating that these sequences were significantly more likely to be detected in soils with pHs of >6. *Methylocystis* spp. occurred in soils with a broad pH range and had little value in sample differentiation. The presence of sequences of USC α and *Methylosinus* spp. was typical for acidic soils (Fig. 2). A contingency analysis on USC α as described above demonstrated that they were more likely to be detected in soils with pHs of <6 than in soils with pHs of >6 (Fisher's exact test, $P = 0.025$), although they were also detected in some higher-pH soils (Table 2).

Statistical analysis with regard to land use or plant cover was not possible because of insufficient data. Nevertheless, it is remarkable that sequences of USC α and USC γ were not detected in any of the four farmland soils analyzed but occurred in 30 of 34 grassland and forest soils. Sequences related to those of *Methylocaldum*, *Methylosinus*, and *Methylocystis* were detected in different soils, independent of land use and plant cover.

Labeling experiments with [^{13}C]methane. Growth in the presence of 1% (vol/vol) labeled $^{13}CH_4$ plus 19% unlabeled CH_4 in the gas headspace led to strong labeling (1,600 to 4,000‰) of all of the PLFAs in *M. trichosporium* KS24b and *Methylocaldum* sp. strain E10a (Fig. 3). Since the separation efficiency was better in GC-MS analysis than in GC-IRMS analysis, separate detection of 18:1ω8c and 18:1ω7c was possible, but the quantification of separate isotope ratios for these two PLFAs in *M. trichosporium* KS24b was problematic because the peaks were not baseline separated in GC-IRMS analysis. Because of the similar labeling intensities of all of the PLFAs, the incorporation of label into the different PLFAs closely reflected the PLFA profiles of the two strains (Fig. 3). 16:1ω7c and 16:0 are the dominant PLFAs in *Methylocaldum* sp. strain E10a, while 18:1ω8c and 18:1ω7c are the main PLFAs of *M. trichosporium* KS24b.

Fresh soil samples were taken in July 2002 from sampling

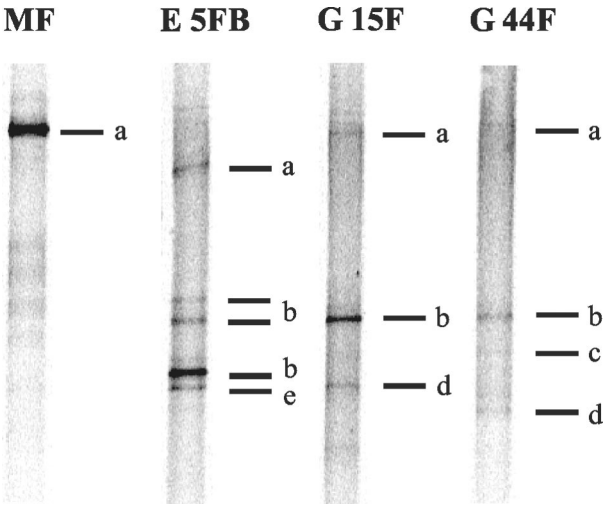

FIG. 4. DGGE banding patterns of *pmoA* PCR products amplified with primers A189f and A682b from soil samples (July 2002) used in the $^{13}CH_4$-labeling experiment. The bands were identified as follows: a, USC α; b, USC γ; c, *Methylocystis* sp.; d, *Methylosinus* sp.; e, unknown (PmoA and AmoA).

sites that had shown high methane oxidation activity and contained sequences of USC γ or USC α or only *pmoA* sequences closely related to *pmoA* of cultivated MB. The MB communities in these samples were similar, but not identical, to those of the earlier samples (Table 2 and Fig. 4). Either the USC γ or the USC α sequences were detected in all of the fresh soil samples. In soil sample MF, only *pmoA* sequences of USC α were detected. Soil samples E 5FB and G 15F showed intense DGGE bands for USC γ in the DGGE gel, compared to the bands of the other detected taxa within the respective samples. Four samples were selected for the $^{13}CH_4$-labeling experiment and a more extensive analysis of methane oxidation kinetics.

As shown in Fig. 5, all four samples showed a typical Michaelis-Menten kinetic. The $K_{m(app)}$ values between 13 and 177 ppmv of CH_4 (17 to 244 nM dissolved CH_4) were typical for the "high-affinity" uptake described by Bender and Conrad (2), and no evidence of additional "low-affinity" activity was observed. The four soil samples were incubated under $^{13}CH_4$ (or unlabeled CH_4) mixing ratios that varied during the incubations from about 10 to 50 ppmv. This value was chosen to be near the estimated $K_{m(app)}$, or within the first-order range of methane uptake. Rate measurements excluded the possibility that shifts occurred within the active community of MB during the incubation. A shift could potentially be caused by induction

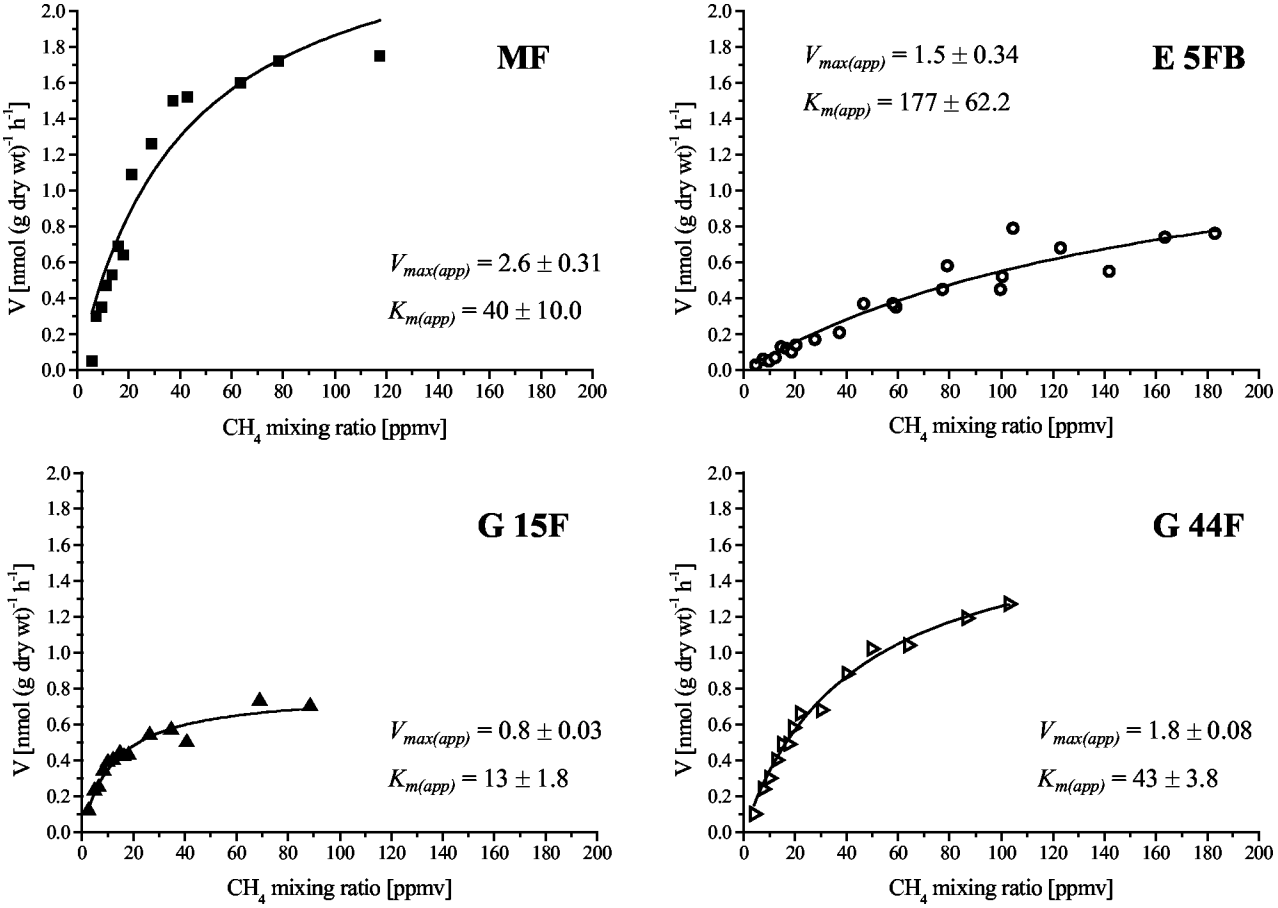

FIG. 5. Methane oxidation kinetics of the soil samples used in the ^{13}C-labeling experiment. Data were fitted to the Michaelis-Menten hyperbolic curve to estimate $V_{max(app)}$ (nanomoles of CH_4 per gram of dry weight per hour ± 1 standard error of fitting) and $K_{m(app)}$ (ppmv of CH_4 ± 1 standard error of fitting). Each data point represents the mean of duplicate samples.

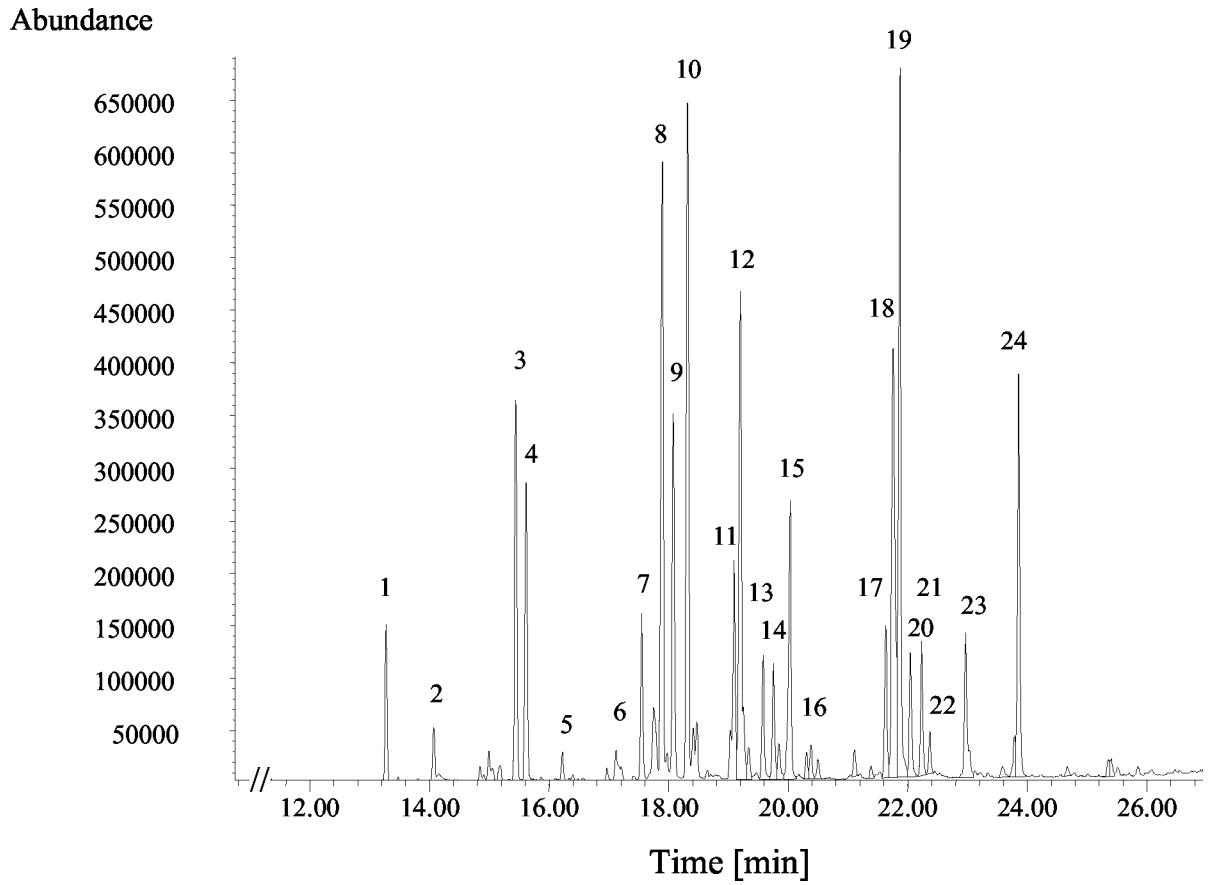

FIG. 6. Partial gas chromatogram of methylated PLFAs detected by GC-MS of soil sample G 44F. Peak numbers indicate PLFAs as follows: 1, i14:0; 2, 14:0; 3, i15:0; 4, a15:0; 5, 15:0; 6, i16:1ω7c; 7, i16:0; 8, 16:1ω7c; 9, 16:1ω5c; 10, 16:0; 11, i17:1ω8c; 12, 16:0ω6m; 13, i17:0; 14, a17:0; 15, 17:0ω7-8cy; 16, 17:0; 17, 18:2ω6,9c; 18, 18:1ω9c; 19, 18:1ω7c; 20, 18:1ω5c; 21, 18:0; 22, 18:1ω7c 7m; 23, 18:0ω8m; 24, 19:0ω7-8cy. Branched fatty acids are indicated by i (iso), a (anteiso), m (methyl), and cy (cyclo).

of methane-oxidizing activity in resting cells or growth of MB. Methane oxidation rates did not increase during the incubation period of 4 weeks (data not shown), indicating that only the already active atmospheric methane oxidizers were labeled.

The total PLFA profiles were very similar in all of the soil samples. The profile of soil sample G 44F is shown as an example in Fig. 6. Saturated and onefold-unsaturated PLFAs with 16 and 18 carbon atoms dominated the profiles. In soil sample MF, incubation with $^{13}CH_4$ led to incorporation of ^{13}C into PLFAs 16:0, i17:0, and 18:1ω7c (Fig. 7). The incorporation of label into 16:0 and i17:0 was much weaker than that into 18:1ω7c. PLFA 18:1ω8c, typical of *Methylosinus* and *Methylocystis* spp., was below the detection limit of the GC-MS method used. The dimethyl disulfide derivatization method used to confirm the positions of double bounds also failed to reveal detectable amounts of 18:1ω8c. The labeled profile in soil MF is most similar to that of type II MB of the genera *Methylocella* and *Methylocapsa*.

A ^{13}C-labeling pattern more typical of type I MB was evident in soil samples E 5FB, G 44F, and G 15F. These profiles were dominated by PLFAs 14:0, 16:1ω7c, and 16:0 (Fig. 7). The labeling intensity was stronger in PLFAs of soil samples G 44F and G 15F than in PLFAs of soil sample E 5FB, which is reasonable considering the relative rates of methane oxidation in these soil samples (Fig. 5).

An additional experiment was performed to exclude the possibility that secondary labeling effects were caused by $^{13}CO_2$ originating through $^{13}CH_4$ oxidation by methanotrophs. Soil samples G 44F and MF were incubated with an amount of $^{13}CO_2$ equal to the total amount of $^{13}CH_4$ consumed in methane incubations. This led to labeling of 18:1 PLFAs in both samples, also of PLFA 19:0ω7-8cy in soil sample MF, and of 18:2ω6,9c in soil sample 44F (Fig. 7). Thus, soil sample G 44F showed different labeling patterns after $^{13}CH_4$ incubation than after $^{13}CO_2$ incubation. In soil sample MF, 18:1ω7c was labeled after incubation with both substrates. However, since the labeling was significantly lower after incubation with $^{13}CO_2$, the labeling of this PLFA after incubation with $^{13}CH_4$ was probably direct labeling of MB and not the result of secondary effects.

DISCUSSION

Upland soils are characterized by high-affinity methane oxidation, with $K_{m(app)}$ values for CH_4 in the nanomolar range (2). The $K_{m(app)}$ values measured in the present work were all similar to values published previously for upland soils (7 to 200 ppmv of CH_4) (2, 3, 9, 15, 20), as were the $V_{max(app)}$ values (2). Although there was considerable variability, all of the soils tested therefore showed kinetic properties typical of atmo-

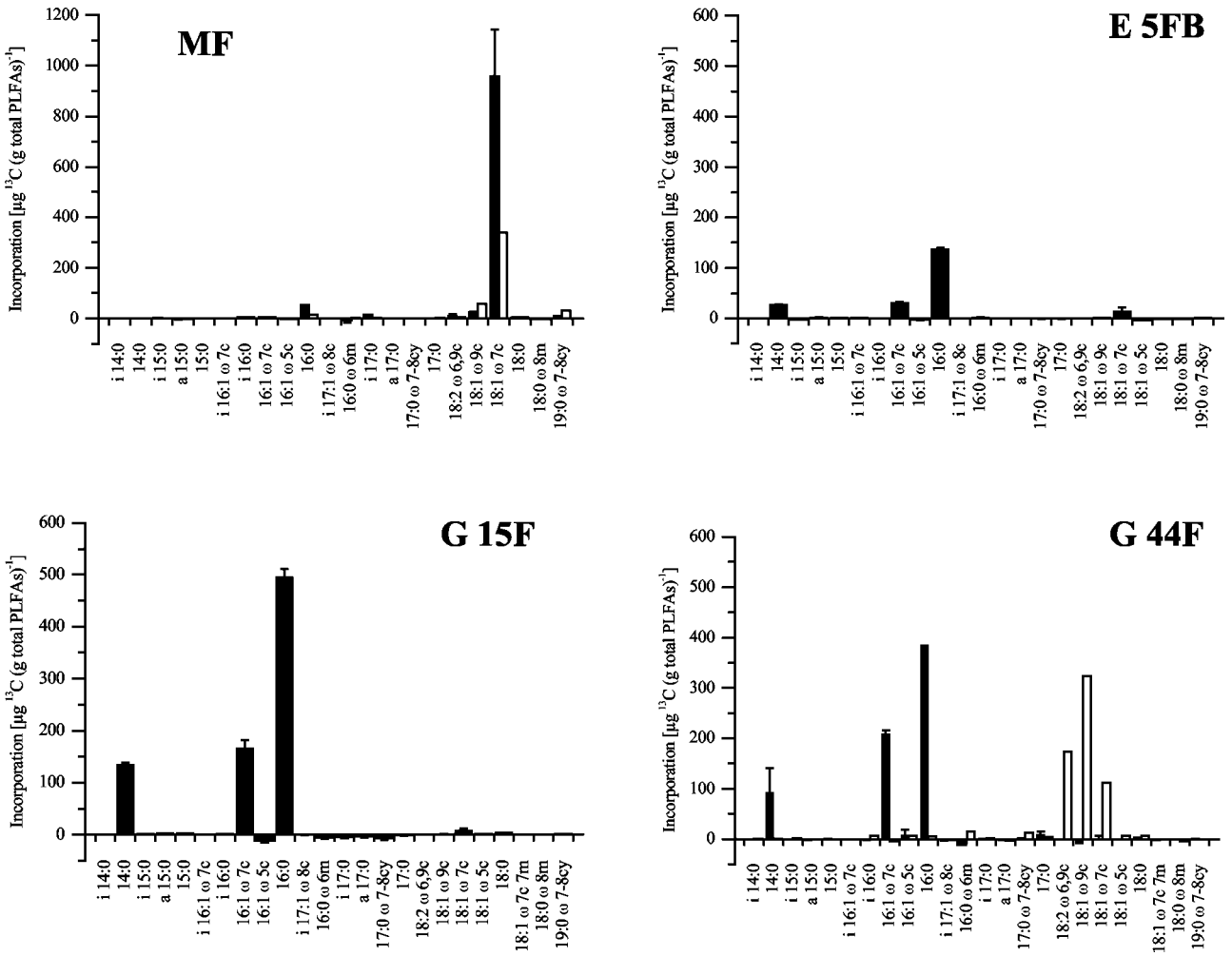

FIG. 7. ^{13}C-labeling patterns of the PLFAs of soil samples incubated under 10 to 50 ppmv of $^{13}CH_4$ (filled bars) or under 225 ppmv of $^{13}CO_2$ (open bars). Incubation with $^{13}CO_2$ was only performed with samples MF and G 44F. For samples incubated with $^{13}CH_4$, data are the mean of triplicates ± 1 standard error of the mean.

spheric methane oxidation. Atmospheric methane oxidation activity in upland soils is dependent on plant cover and land use, such that rates in woodland > rates in grassland > rates in farmland (48). Although not statistically tested, our data confirm this trend insofar as the highest a^0_s values (which represent the initial slope of the hyperbolic curve at low CH_4 mixing ratios) were observed in forest soils. Where several adjacent sampling sites with different land uses were analyzed, a^0_s decreased with increasing anthropogenic disturbance from woodland to pasture to cropland: specifically, in samples E 26F, E 26W, and E 26A; E 56F, E 56W, and E 56A; or G 15F, G 15W, and G 15A (Table 1).

PmoA sequence analysis revealed that the soils contained MB closely related to cultivated species and also some only distantly related to known MB. We did not detect *pmoA* sequences related to those of the genera *Methylomonas*, *Methylomicrobium*, *Methylobacter*, and *Methylococcus*, which have been detected in forest soils from Denmark and the United Kingdom (4, 42), but we did commonly find *pmoA* sequences closely related to *Methylocaldum*, *Methylocystis*, and *Methylosinus*. Sequences of USC α were very common in our soils, as expected from previous studies (4, 23, 28, 32). This cluster has been postulated to represent as yet uncultivated MB active at consuming atmospheric methane (28). We also frequently detected *pmoA* sequences in a cluster (USC γ) that grouped most closely with sequences of type I MB but had only low identity values (<75%) with PmoA of cultivated species. A few other sequence types were detected, but only infrequently.

Sequences of USC α or USC γ have never been detected in wetland soils. The occurrence of these sequences seems to be restricted to upland soils that consume atmospheric methane. A correlation between the occurrence of these groups and the soil pH value was also evident from our data. The USC γ sequences were significantly more likely to be detected in soils with pH values of >6.0 than in more acidic soils, whereas the opposite trend was evident for USC α. Different MB are known to have different pH optima (11, 21), but pH can only be considered an indicator rather than a cause in this study. The neutral or alkaline soils studied were primarily Leptosols derived from a calcareous substrate, and some chemical factor other than pH may be decisive in determining whether a particular MB group is present. Alternatively, secondary pH effects, for example, on micronutrient availability, may be critical.

The $^{13}CH_4$-labeling experiment was performed to identify the

active MB in soils in which *pmoA* sequences of USC γ, of USC α, or of cultivated MB were detected. A CH_4 mixing ratio (10 to 50 ppmv) near the $K_{m(app)}$ for methane oxidation of the soils was used for incubations. Control measurements ensured that only the already active high-affinity population was labeled and that secondary labeling effects of $^{13}CO_2$ were minimal. In soil sample MF, labeling of PLFAs 16:0, i17:0, and 18:1ω7c was obtained after $^{13}CH_4$ incubation. PLFA 18:1ω7c is the main fatty acid of *M. acidiphila* and *Methylocella* spp. (11, 14). PFLAs typical of type I MB (e.g., 14:0 and 16:1ω7c) or of other type II MB (e.g., 18:1ω8c) were not detectably labeled. A labeling pattern similar to that of soil sample MF was previously found in another upland soil (8), although a different branched 17:0 PLFA was labeled instead of i17:0. Fatty acid i17:0 has been found only in small amounts (<0.6%) in a few cultivated MB (7, 11), and the labeling of branched 17:0 PLFAs was previously interpreted to mean that active MB in soil are unusual compared to known species (8). This may also be the case in soil sample MF; however, such a conclusion is dangerous because i17:0 accounted for only a tiny percentage of the ^{13}C incorporated. PLFA-labeling profiles similar to that of soil sample MF were also obtained by incubation of six acidic upland soil samples under ^{14}C-labeled methane, followed by scintillation counting of fractionated FAMEs (28, 44). Although this fractionation procedure resulted in limited separation efficiency, one labeled PLFA in soil sample MF would fall within each of the labeled fractions described in these previous studies (28, 44). The use of labeled PLFAs as chemotaxonomic marker molecules correlates well with the *pmoA* recovery in soil sample MF. The only *pmoA* sequences detected in soil sample MF belonged to USC α, a unique cluster of which the type II MB *M. acidiphila* is the closest relative. Taken together, these results strongly support the hypothesis of Holmes et al. (28) that as yet uncultivated methanotrophic *Alphaproteobacteria* possessing *pmoA* sequences of USC α are responsible for atmospheric methane oxidation in some upland soils.

On the other hand, the more neutral soil samples E 5FB, G 44F, and G 15F showed PLFA labeling patterns clearly distinct from that of soil sample MF. The combination of labeled 14:0 and 16:1ω7c suggests that MB more closely related to cultivated type I MB were active in these soil samples. PLFA 16:1ω7c contributes 8 to 57% of the total amount of PLFAs in all characterized type I MB but much less in type II MB (<9%) (7, 40). Fatty acid 14:0 is abundant only in type I MB. It contributes up to 25% of the total PLFA content of *Methylomonas* spp. and smaller amounts (1 to 10%) to that of other type I MB (5, 7). Only traces (<0.1%) have been found in *Methylosinus* spp. and *Methylocystis* spp. (7, 40), and it is undetectable in *Methylocella* spp. and *M. acidiphila* (11, 14).

At the very least, the labeling results indicate that different MB are active in different soils and include strains related to either type I (*Gammaproteobacteria*) or type II (*alphaproteobacteria*) MB. The most likely explanation for the combined *pmoA* and PLFA data for soil samples E 5FB, G 44F, and G 15F is that unknown methanotrophic *Gammaproteobacteria* possessing *pmoA* sequences of USC γ are the most active MB. This *pmoA* sequence type was detected in all three soils and was clearly the dominant *pmoA* product obtained from two. It was the only *pmoA* sequence detected that was closely related to *pmoA* sequences of type I MB, and it is therefore likely that the organisms containing these sequences were the same MB labeled with $^{13}CH_4$. However, it cannot be excluded that another type I MB that we did not detect was present and active. We have observed, for example, that the *pmoA* genes of several *Methylobacter* strains are poorly amplified with the standard primer sets used here (data not shown). Sequences from USC α and sequences related to those of *Methylocystis* and *Methylosinus* spp. were also detected in one or all of the E 5FB, G 44F, and G 15F soil samples, but labeled PLFAs 18:1ω8c and 18:1ω7c indicative of *Methylocystis* and *Methylosinus* spp. (7, 40), and presumably also of USC α, were not found. This finding demonstrates that not all of the methanotrophs detectable in an upland soil sample are equally involved in the process of methane oxidation. USC γ was only detected in soils with pHs of >6 and may therefore represent a neutrophilic or alkalophilic MB. This would explain why earlier experiments with acidic soils failed to show a PFLA-labeling pattern like that observed in the more neutral soil samples E 5FB, G 44F, and G 15F.

The comparison of the *pmoA* data and the PLFA patterns in this and earlier studies is based on the usefulness of both biomolecules as phylogenetic markers. Although *pmoA* is, for the most part, an excellent phylogenetic marker, two studies have demonstrated that some caution is necessary in the interpretation of *pmoA* sequences. The recently described methanotrophic isolates K3-16 and K1-8 are related to *Methylosinus* and *Methylocystis* on the basis of 16S rRNA sequence comparison but only distantly related on the basis of *pmoA* sequence comparison (41). As well, a second *pmoA*-like gene (*pmoA2*) has recently been found in some strains of *Methylosinus* and *Methylocystis*. These sequences have <80% identity to the previously known *pmoA* gene (*pmoA1*) sequences of the respective organisms (16). Together, these two studies indicate that a *pmoA*-based phylogeny does not perfectly reflect the 16S rRNA-based phylogenetic affiliation of MB and that novel clusters of *pmoA* sequences do not necessarily indicate that novel groups of uncultivated MB exist.

Nevertheless, we feel that the most likely explanation for our data is that *pmoA* sequences of USC α and USC γ belong to unknown groups of MB active in upland soils. No other *pmoA* gene sequences were detected together with those of USC α in soil samples MF, WO DFT, VA DFH, BB NNW, and G 13FH. The USC γ sequences were detected together with sequences of other genera; however, in the samples used for ^{13}C labeling, none of the other MB detected would be expected to give a labeling profile typical of type I MB. Taken together, the data presented here indicate that different groups of MB are responsible for atmospheric methane uptake in different soils and that the MB in neutral-to-alkaline Leptosols are most closely related to type I MB of the *Gammaproteobacteria*. Indirect evidence from other studies has suggested that MB closely related to cultivated species may also be active in certain upland soils (15, 29), so in all, many diverse species of MB may be involved in the process of atmospheric methane oxidation.

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