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ORIGINAL ARTICLE

Methanol oxidation by temperate soils and environmental determinants of associated methylotrophs

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The role of soil methylotrophs in methanol exchange with the atmosphere has been widely overlooked. Methanol can be derived from plant polymers and be consumed by soil microbial communities. In the current study, methanol-utilizing methylotrophs of 14 aerated soils were examined to resolve their comparative diversities and capacities to utilize ambient concentrations of methanol. Abundances of cultivable methylotrophs ranged from 10^6–10^8 g^{-1}_{soilDW}. Methanol dissimilation was measured based on conversion of supplemented ^{14}C-methanol, and occurred at concentrations down to 0.002 μmol methanol g^{-1}_{soilDW}. Tested soils exhibited specific affinities to methanol ($a^0_s = 0.01\ d^{-1}$) that were similar to those of other environments suggesting that methylotrophs with similar affinities were present. Two deep-branching alphaproteobacterial genotypes of *mch* responded to the addition of ambient concentrations of methanol (≤0.6 μmol methanol g^{-1}_{soilDW}) in one of these soils. Methylotroph community structures were assessed by amplicon pyrosequencing of genes of mono carbon metabolism (*mxaF*, *mch* and *fae*). *Alphaproteobacteria*-affiliated genotypes were predominant in all investigated soils, and the occurrence of novel genotypes indicated a hitherto unveiled diversity of methylotrophs. Correlations between vegetation type, soil pH and methylotroph community structure suggested that plant–methylotroph interactions were determinative for soil methylotrophs.

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Introduction

Aerobic methylotrophic bacteria in soils have been recognized as drivers of methane fluxes in terrestrial ecosystem (Dunfield, 2007; Trotsenko and Murrell, 2008; Conrad, 2009; Kolb, 2009a). However, their role in methanol exchange with the atmosphere has widely been overlooked, even though the annual global emission rate (5 Tmol per year) of methanol is close to that of methane (that is, 10 Tmol per year; Jacob *et al.*, 2005), and most methylotrophs utilize methanol (Lidstrom, 2006; Chistoserdova *et al.*, 2009; Kolb, 2009a).

Methanol is distantly the second most abundant organic compound in the atmosphere (0.1–10 p.p.b.) after methane (1800 p.p.b.), but it is chemically more reactive than methane. Tropospheric methanol reacts with nitrogen oxides to produce HO_x radicals and affects the oxidizing capacity of the troposphere and ozone formation. The main source of atmospheric methanol is plant biomass (Galbally and Kirstine, 2002; Jacob *et al.*, 2005). Methanol can be released from the methoxy groups of pectin and lignin (Donnelly and Dagley, 1980; Schink and Zeikus, 1980; Fall and Benson, 1996; Warneke *et al.*, 1999). Estimates of global rates of methanol emission based on plant biomass production are considerably higher (26 Tmol per year) than the observed rates (Galbally and Kirstine, 2002; Jacob *et al.*, 2005; Kolb, 2009a) suggesting that methylotrophs of terrestrial ecosystems likely consume methanol and thereby partially mitigate its emission into the atmosphere (Kolb, 2009a). However, quantitative information on this activity in terrestrial ecosystems is lacking. As methanol is plant-derived in terrestrial ecosystems, plant surfaces, such as

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leaves, can be considered as favored habitats of aerobic methylotrophs. It has indeed been demonstrated that leaf surfaces of various temperate plant species are inhabited by active methylotrophs, which constitute about 14–20% to the total microbial community of the phyllosphere (Holland *et al.*, 2002; Delmotte *et al.*, 2009; Fedorov *et al.*, 2011; Wellner *et al.*, 2011; Mizuno *et al.*, 2012). However, the upper soil layers of forests and grasslands are also well supplied with oxygen, and belowground parts of plants are likely sources of methanol.

Utilization of methanol requires unique metabolic pathways that differentiate methylotrophs from other aerobic heterotrophs. Genes encoding enzymes associated with mono carbon metabolisms are therefore suitable targets for detecting methylotrophs in the environment (McDonald *et al.*, 2008). Methylotrophs oxidize methanol sequentially to carbon dioxide. The pyrroloquinolinequinone-dependent methanol dehydrogenase (PQQ MDH) (encoded by *mxaFI*) catalyzes the first step of methanol dissimilation in various Gram-negative bacteria (Chistoserdova *et al.*, 2009). To date, *mxaF* is the only targeted gene in environmental studies of methylotrophs that encodes for a methanol-oxidizing enzyme (McDonald *et al.*, 2005, 2008). A homolog (*mdh2*) exists in *Burkholderiaceae*, but *mdh2* is not as widely distributed within methylotrophs as *mxaF* (Kalyuzhnaya *et al.*, 2008). *xoxF*, a similar gene, occurs in all known methylotroph genomes (Chistoserdova, 2011). Its gene product XoxF oxidizes *in vitro* methanol (Schmidt *et al.*, 2010). XoxF is needed for PQQ MDH activity and is involved in the regulation of PQQ MDH in *Methylobacterium extorquens* AM1. Whether XoxF can also function as an alternative MDH *in vivo* is unknown and currently under debate (Skovran *et al.*, 2011). In many Gram-negative methylotrophs, formaldehyde is transferred to tetramethanopterine (H_4MPT) by the formaldehyde-activating enzyme (encoded by *fae*), and oxidized to formate by reactions that employ H_4MPT-dependent enzymes (Vorholt *et al.*, 1999). One of those enzymes, methenyl-cyclohydrolase (encoded by *mch*), has been successfully used to resolve methylotrophic community structure and diversity in a lake (Kalyuzhnaya *et al.*, 2004, 2005).

Phylogenetic identities of methylotrophs in soils that utilize methanol at *in situ* concentrations have not been resolved. Taxa that assimilated supplemented [^{13}C]-methanol in an aerated forest soil have been identified (Radajewski *et al.*, 2002), but current knowledge is based on pure cultures and soil experiments (Radajewski *et al.*, 2002; Kolb, 2009a), in which methanol was supplemented in millimolar concentrations that likely do not occur in soil. Substrate affinities (that is, half-saturation constant, K_m) of methanol dehydrogenases of soil-derived methylotrophs are above 1000 nmol l^{-1} (Arfman *et al.*, 1989; Hektor *et al.*, 2002; Nojiri *et al.*, 2006). Recent measurements of K_m (K_m; 9.3 nmol l^{-1}) in marine surface water samples (Dixon *et al.*, 2011) suggest that unknown and low methanol concentration-adapted methylotrophs utilize methanol in marine ecosystems. Such information is missing for terrestrial ecosystems, which are not only the main source of atmospheric methanol but are also a sink for it (Karl *et al.*, 2005; Schade *et al.*, 2011).

The objectives of the current study were (a) to determine substrate affinities for aerobic methanol dissimilation in aerated soils, (b) to identify genotypes that respond to micro molar concentrations of methanol in aerated soils, (c) to assess community structures of methylotrophs that were detected by *mxaF*, *mch* and *fae* pyrosequencing and cultivation in aerated forest and grassland soils, and (d) to relate site-specific environmental parameters with methylotroph community structures.

Materials and methods

Study sites, sampling and sample preparation

Top mineral soils of 14 different temperate, aerated grassland and forest soils in Germany were investigated (Table 1). The grassland soils FG, OG and HEG 6 were used in experiments in which methanol dissimilation rates and Michaelis–Menten kinetics were determined. In case of FG and OG, top mineral soil of three subsites was sampled and pooled. Stones and other large materials were removed manually. The grassland soils AEG 2, AEG 7, HEG 6, HEG 9, SEG 2 and SEG 6, and the forest soils AEW 5, AEW 8, HEW 5, HEW 12, SEW 5 and SEW 9 were a subset of sampling plots that are distributed in three regions over a transect from northeast to southwest of Germany and set up within a joint research project that assesses biodiversity and soil parameters at about 3000 sampling sites (Fischer *et al.*, 2010). The sites used in the current study represented grasslands and forests in three German regions (that is, Schorfheide Chorin, Hainich and Schwäbische Alb) with both high and low land use intensity. On several occasions from April 2008 to October 2009 (Table 1), five soil cores were taken per site, and A horizon (that is, top mineral soil) material was manually prepared and pooled. A detailed sampling schedule can be found in Supplementary Table S1. All soil samples were transported on ice and immediately frozen at $-80\,^{\circ}$C for molecular analyses. Soils (FG, OG and HEG 6) that were used for methanol oxidation rate measurements were stored at 2 °C for further analysis.

Methanol Oxidation Rates in Grassland soils FG, OG and HEG 6

1) Measuring Michaelis-Menten kinetics for samples of soils FG and HEG 6. One gram fresh weight of soil was suspended in sterile water adding up to a volume of 5 ml in gas-tight tubes (Bellco Glass Inc., Vineland, NJ, USA). Slurries were supplemented

Table 1 Description of analyzed soils, including sampling period, and assignment to conducted analyses

Soil	*Soil type*[a]	*Location*	*Vegetation*[b] *(land use intensity)*[c]	*pH*[d]	NO_3^-/NH_4^+ ($\mu g\, g_{TC}^{-1}$)	*Sampled*[e]	*Analysis*
FG	Cambisol	N50°08′ E11°52′	Grassland (ni)	6.0	—	2010	MeOH dis
OG	Histosol	N49°57′37″ E11°35′42″	Grassland (ni)	7.0	—	2010	MeOH dis
AEG 2	Leptosol	N48° 22′ 36.686″ E9° 28′ 22.023″	Grassland (i)	6.9	2.8/23.8		Pyro Isol MPN
AEG 7	Leptosol	N48° 23′ 29.116″ E9° 22′ 36.65″	Grassland (ni)	7.6	1.8/23.2		Pyro MPN
AEW 5	Cambisol	N48° 25′ 10.626″ E9° 24′ 52.854″	Forest (i)	5.6	1.3/24.7		Pyro MPN
AEW 8	Cambisol	N48° 22′ 57.322″ E9° 22′ 56.584″	Forest (ni)	6.4	2.2/25.0		Pyro[f] Isol MPN
HEG 6	Stagnosol	N51° 12′ 53.766″ E10° 23′ 28.395″	Grassland (i)	6.5	0.4/20.2		MeOH dis Pyro TRFLP Isol MPN
HEG 9	Stagnosol	N51° 13′ 26.031″ E10° 22′ 50.834″	Grassland (ni)	7.0	0.3/19.7	2008 2009	Pyro Isol MPN
HEW 5	Luvisol	N51° 15′ 49.961″ E10° 14′ 27.448″	Forest (i)	5.4	1.0/25.0		Pyro Isol MPN
HEW 12	Luvisol	N51° 6′ 2.477″ E 10° 27’ 18.659”	Forest (ni)	4.8	0.6/20.9		Pyro Isol MPN
SEG 2	Histosol	N53° 5′ 21.505″ E13° 58′ 48.169″	Grassland (i)	7.5	1.6/24.9		Pyro
SEG 6	Histosol	N53° 6′ 12.583″ E13° 37′ 22.2″	Grassland (ni)	5.8	1.6/23.7		Pyro MPN
SEW 5	Cambisol	N53° 3′ 25.321″ E 13° 53′ 7.318″	Forest (i)	4.0	0.6/16.4		Pyro[g,f,h] Isol MPN
SEW 9	Cambisol	N53° 2′ 40.513″ E13° 48′ 36.371″	Forest (ni)	4.5	0.4/15.2		Pyro[g] Isol MPN

Abbreviations: Isol, isolation of pure cultures; MeOH dis, determination of methanol dissimilation based on the amount of $^{14}CO_2$ produced from ^{14}C-methanol; MPN, most probable numbers of methanol-utilizing methylotrophs (details in Supplementary Information ‘Methods’); Pyro, pyrosequencing of genes *mxaF*, *fae* and *mch*; TRFLP, terminal restriction fragment length polymorphism analysis with *mch*.
[a]Data were taken from Fischer *et al*., 2010.
[b]Grassland, temperate grassland with various grasses and weeds; Forest, mixed hardwood forests that were dominated by beech (Fagus sylvatica L.).
[c]i, intense land use (i.e., grasslands were regularly fertilized and forests were regularly harvested); ni, non-intense land use (i.e., grasslands no fertilized and forests were not subjected to land use since at least 60 years).
[d]pH in water; mean values from three independent measurements.
[e]Detailed sampling scheme is given in Supplementary Table S1.
[f]In these soils amplification of *mch* was not successful.
[g]In these soils amplification of *mxaF* was not successful.
[h]In these soils amplification of *fae* was not successful.

with ^{12}C-methanol with six different concentrations between 0.001 and 250 $\mu mol\, g_{soil\ fresh\ weight}^{-1}$. Traces of ^{14}C-methanol (1.2 $nmol_{soil\ fresh\ weight}^{-1}$) were added. Slurries were incubated at room temperature, and formed carbon dioxide (CO_2) was trapped in two sequential sodium hydroxide traps over a period of 3 days. CO_2 traps consisting of tubes containing sodium hydroxide (per trap tube 1 ml NaOH (1 M) were connected to the slurries with gas-tight tubing. Flushing with sterile air was used to drive $^{14}CO_2$ into the traps. Hundred-microliter aliquots were measured by scintillation counting (LS 6500, Beckman Coulter GmbH, Krefeld, Germany) after overnight incubation in the scintillation cocktail (Ecolume Liquid Scintillation Cocktail, MP Biomedicals Germany GmbH, Eschwege, Germany). Sum of counts per minute from both traps, and the specific activity of ^{14}C-methanol (Biotrend GmbH, Köln, Deutschland) were used to calculate formed $^{14}CO_2$. Based on the proportion of $^{14}CO_2$ to $^{12}CO_2$, the total amount of formed CO_2 was determined. Production of CO_2 was used to estimate the dissimilated amount of methanol. All measurements were conducted in experimental duplicates. As controls, abiotic formation of $^{14}CO_2$ was measured in treatments with sterile water that was supplemented with ^{14}C-methanol, and in soil slurries with radioactive methanol that were biologically inactivated by adding 10 mM potassium cyanide (Watanabe *et al*., 1996). Maximal velocity (V_{max}) and K_m were

determined based on calculated oxidation rates of ^{14}C-methanol at different concentrations that were non-linearly fitted (SigmaPlot, version 10.0, Systat Software GmbH, Erkrath, Germany; Segel, 1993).

2) Localization of methanol oxidation activity in soil OG. Washed roots, root-free soil from soil OG and HEG 6, and a sterilely grown grassland plant (*Arabidopis thaliana*, cultivation procedure in Supplementary Information 'Methods') were supplemented with total concentrations of ^{14}C- and ^{12}C-methanol of 51 nmol $g^{-1}_{freshweigt}$. The difference in produced $^{14}CO_2$ between the start of the experiment and after 13 days was determined. For each sample, a biologically inactivated treatment with sodium cyanide was set up.

Terminal restriction fragment length polymorphism of mch genes in samples of ^{14}C-methanol-supplemented slurries

DNA was extracted from slurry samples of the experiments, in which Michaelis–Menten kinetics were determined in soils FG and HEG6, according to a previously published protocol (Stralis-Pavese *et al.*, 2004). Sufficient amounts of DNA could not be extracted from slurry samples of FG, and OG, and amplification of genes *mxaF* and *fae* was not successful with extracts of slurries of soil HEG 6. Primer mch-2a was covalently labeled with fluorescent dye IRDye 681 (Microsynth AG, Lindau, Germany). PCR products were digested with restriction enzyme *Bsl*I (New England Biolabs GmbH, Frankfurt a.M., Germany), and separated on sequencer (NEN4300, Licor GmbH, Bad Homburg, Germany) procedure and settings were the same that were used in a previous study, in which 16S ribosomal RNA (rRNA) gene terminal restriction fragment (TRF) length polymorphism was conducted (Schellenberger *et al.*, 2010). Retrieved TRF patterns were used to identify those TRFs that were enriched in methanol-supplemented slurries. *mch* was also amplified using the same primers as for TRF length polymorphism but without fluorochrome label. These amplicons were pooled, and separated by TA cloning into competent *Escherichia coli* cells (LGC Genomics GmbH, Berlin, Germany). Ninety-six inserts in transformed plasmid vectors were sequenced (LGC Genomics GmbH). Sequence information was used to assign TRFs to genotypes of *mch* (TRFCUT; Ricke *et al.*, 2005). Briefly, *mch* sequences were aligned and hypothetical TRFs were determined. Measured TRFs were compared with predicted TRFs to assign TRFs to *mch* sequences.

Most probable numbers of methanol-utilizing methylotrophs

Viable cell numbers of methanol-utilizing, aerobic methylotrophs were determined by most probable number (MPN) technique (Alef, 1991). From soils that were sampled in April of 2008 and 2009 (Table 1, Supplementary S1 and S3), MPNs were determined in mineral media (M1; Dedysh *et al.*, 1998) or 125 (DSMZ *Methylobacterium* medium, Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). Media were supplemented with 10 mM of methanol at pH 3.1 or 6.8, with or without additional nitrate (0.5–1.0 mM) and with or without vitamine solution (Atlas, 1993). Serial dilutions of soil (10^2 up to 10^9 fold) were inoculated in 10 replicates of fresh medium in 96-well microtiter plates (Sarstedt GmbH, Nümbrecht, Germany). For each plate, a row of wells were filled with sterile medium as controls. Plates were incubated at 20 °C for up to 72 days (details in Supplementary Information 'Methods'). Wells were regularly checked for growth until no change in number of growth-positive wells was observed. Growth was detected based on turbidity measured at 660 nm (μQuant Universal Microplate Spectrophotometer, BIO-Tek Instruments GmbH, Bad Friedrichshall, Germany). Measured optical density was corrected for values at the start of incubation to account for different amounts of inoculated cells. Based on the variability of data, a change of 0.03 in OD_{660} was interpreted as growth. Determined MPNs (based on tables in Alef, [1991]) per milliliter of inoculum were converted to gram dry weight of soil (Supplementary Table S2). These MPNs were correlated with environmental parameters in subsequent statistical analyses.

Amplicon pyrosequencing of mxaF, mch and fae

DNA that was used for barcoded amplicon pyrosequencing was extracted from 0.3 g of soil according to a published protocol (Stralis-Pavese *et al.*, 2004). The procedure includes lysis by beat beating. Extracted DNA was dissolved in 100 μl DNAse and RNAse-free water, and stored at −80 °C for further analysis. Undiluted or one-hundred-fold diluted DNA extract was used in subsequent amplification of *mxaF*, *fae* and *mch* genes. The used gene-specific primers were tagged at the 5′ terminus with a six-nucleotide barcode that encoded for a specific soil. *mxaF* was amplified with primers 1003f (5′-GCGGCACCAACTGGGGCTGGT-3′) and 1555r (5′-CATGAABGGCTCCCARTCCAT-3′) (McDonald and Murrell, 1997; Neufeld *et al.*, 2007), *fae* was amplified with primers fae1f (5′-GTCGGCGACGG CAAYGARGTCG-3′) and fae1r (5′-GTAGTTGWAN TYCTGGATCTT-3′) (Kalyuzhnaya *et al.*, 2004), and *mch* was amplified with primers mch-2a (5′-TGCC TCGGCTCKCAATATGCYGGBTGG-3′) and mch-3 (5′-GCGTCGTTKGTKCKBCCCAT-3′) (Vorholt *et al.*, 1999). For amplification of *mch* and *fae*, 50 μl reactions were performed on a Primus 96 thermocycler (PeqLab Biotechnologie GmbH, Erlangen, Germany), which contained 25 μl of Master Amp PCR premix (2.5-fold; Epicentre Biotechnologies Inc., Madison, WI, USA), 0.15 μl of Taq DNA

polymerase (5 U μl^{-1}; Invitrogen GmbH, Karlsruhe, Germany), 5 µl of each primer solution (10 µM), 5 µl of template DNA and 9.85 µl of RNase- and DNase-free water. *mch* and *fae* were amplified according to the following temperature program, that is, intial denaturation (95 °C, 1 min) followed by a touch-down step (denaturation, 95 °C, 0.5 min; annealing, 55 down to 45 °C, 0.5 min; and elongation, 72 °C, 1.3 min), followed by 20 cycles of amplification (denaturation, 95 °C, 0.5 min; annealing, 45 °C, 0.5 min; and elongation, 72 °C, 1.3 min). The program was finished with a single elongation step (72 °C, 10 min). As *mxaF* could not reliably amplified with the above described reactions mix, the reaction mix 50 µl contained 10 µl of Phusion HF Buffer (fivefold; NEB), 0.5 µl of Phusion DNA polymerase (NEB), 1.5 µl of dimethyl sulphoxide (100%), 1.5 µl of $MgCl_2$ (50 mM; NEB), 4 µl of didesoxy-nucleotide mix (2.5 mM; NEB), 3 µl of each primer solution (10 µM), 1 µl of template DNA, and 25.5 µl of RNase- and DNase-free water. The temperature program started with a single denaturation step (98 °C, 1 min), followed by 35 amplification cycles (denaturation, 98 °C, 0.6 min; combined annealing and elongation, 72 °C, 0.5 min). The protocol ended with a final elongation step (72 °C, 6 min).

PCR products were cut out from agarose gels, and were extracted with the Montage Gel Extraction Kit (Millipore GmbH, Schwalbach, Germany). Amplicon mixtures were treated with PreCRepair Mix (NEB) to eliminate possible PCR-blocking DNA damage that might have occurred during gel purification or storage of amplicons, and were purified by isopropanol precipitation at −20 °C overnight. Purified and dried DNA was dissolved in 20 µl of RNAse- and DNAse-free water, and amplicons of *mch* and *fae* were pooled and then pyrosequenced at the Göttingen Genomics Laboratory using the Roche GS-FLX 454 Sequencer and GS FLX Titanium series reagents according to manufacturers recommendations (Roche Diagnostics GmbH, Mannheim, Germany). In brief, an emulsion PCR was performed after ligation of A (5′-CGTATCGCCTCCCTCGCGCCAT CAG-3′) and B (5′-CTATGCGCCTTGCCAGCCCGCT CAG-3′) sequencing adaptors. Subsequently, beads were transferred on a picotiter plate, and were sequenced. *mxaF* was independently sequenced from *mch* and *fae* as the number of reads was not sufficient when *mxaF* amplicons were mixed up with *mch* and *fae* PCR products.

Quality filtering and clustering of mxaF, mch and fae amplicon pyrosequencing reads

The raw reads were processed using AmpliconNoise (Quince *et al.*, 2011). In summary, pyrosequencing flowgrams that had at least one flow with signal intensity between 0.5 and 0.7 or a cycle of four nucleotide flows (TACG) that failed to give a signal >0.5 before cycle number 400 (both indicative of noise) were discarded, and all reads were truncated at cycle number 600. Pyrosequencing noise was subsequently removed using PyroNoise (Quince *et al.*, 2011) using the default settings, and PCR noise was removed using SeqNoise ($\sigma s = 0.033$ and $cs = 0.08$) (Quince *et al.*, 2011) after truncation at 400 bp to reduce noise further.These data were then subjected to sorting, clustering and BLAST analysis with Jaguc (Version 2.1; Nebel *et al.*, 2011). Only reads starting at the forward primer and being longer than 200 bp were further analyzed. All reads from all soils were then clustered and identified using BLAST (Altschul *et al.*, 1990). To get reliable BLAST search result, the following non-default parameter settings were chosen (blastn, non-default expect threshold value was 100). Genotypes of non-target genes were removed before further analysis. Sequences were affiliated to genotypes, that is, operational taxonomic units (OTUs), based on similarity cutoff values of 77%, 80% and 80% for *mxaF*, *mch* and *fae*, respectively, which were determined using the average neighboring method (Nebel *et al.*, 2011). Barcode identifiers were used to determine the relative frequency of a genotype in amplicons from a certain soil after clustering. Similarity cutoff values were determined as follows. As the data basis for calculation of species-level cutoff for *mxaF* was sufficient (that is, 60 strains with both gene sequences were available), a comparison of pair-wise similarities of 16S rRNA genes that were related to pair-wise similarities of corresponding *mxaF* similarities revealed a species-level similarity cutoff of 77% for *mxaF* that corresponds to 97% similarity on 16S rRNA gene level (Supplementary Information 'Methods' and Supplementary Figure S1a). There was no sufficient information of 16S rRNA sequences and affiliated *mch* or *fae* sequences. Thus, quality-filtered reads of *mch* and *fae* from all soils were clustered using decreasing cutoff values for gene similarities, based on which sequences were clustered with Jaguc. Maximally high cutoff values were chosen, at which the number of retrieved genotypes stayed constant (Supplementary Information 'Methods' and Supplementary Figure S1b).

Reconstruction of MxaF, Mch and Fae phylograms

From each OTU, one representative amino-acid sequence was aligned with known reference sequences (MEGA5, Tamura *et al.*, 2011), and used to reconstruct neighbor-joining trees (1000 replicated calculations, Saitou and Nei, 1987). The trees were used to provide phylogenetic assignments to novel genotypes. All presented sequences of rarified data sets were deposited at the European Bioinformatics Institute, that is, accession numbers HE970319–HE970434.

Statistical analyses of community structure (mxaF, mch and fae) and abundance (MPN) data sets

Genotypes (that is, number of OTUs) coverages were always above 98% (Supplementary Table S5). As

Table 2 Kinetic parameters of grassland soils OG, FG and HEG 6

Soil	*Methanol dissimilation rate at 74 nmol methanol* g_{DW}^{-1} *(μmol* $g_{DW}^{-1}d^{-1}$*)*[a]	v_{max} *(mM)*[b]	K_m *(mM)*[b]	a_s^o (d^{-1})
OG Slurry 1	0.001	—	—	—
OG Slurry 2	0.001	—	—	—
FG Slurry 1	0.005	0.09 (<0.01)	1.31 (0.06)	0.07
FG Slurry 2	0.007	0.16 (0.01)	4.0 (0.7)	0.04
HEG Slurry 1	0.0006	0.39 (0.01)	236.3 (21.0)	0.01
HEG Slurry 2	0.0006	0.34 (0.01)	200.9 (16.0)	0.01

Abbreviations: a_s^o, specific affinity; K_m, Michaelis–Menten constant; v_{max}, maximal velocity; —, not measured.
[a]74 nmol methanol per gram dry weight of soil corresponds to 10 μM in slurry.
[b]In parentheses, standard errors of predicted parameters are given (a_s^o is the quotient of v_{max} divided by K_m, thus no error is given). Regressions are presented in Supplementary Figure S2.

numbers of reads per gene and soil were variable (Supplementary Table S5), data sets for each gene were rarefied using QIIME (Caparose *et al*., 2010). Sample size was adjusted to the soil with lowest number of reads (Supplementary Table S5). Normal distribution of the data set was tested for the 10 most abundant OTUs of each gene by the Shapiro–Wilk test (Royston, 1995) using the software OriginPro 8G SR4 (OriginLab Corporation, Northhampton, MA, USA). As most of the tested data were not normally distributed, canonical correspondence analyses (CCA) with log-transformed data were conducted. The effect of genotype and MPN data on the environmental parameters (that is, vegetation type (forest and grassland), land use intensity (managed and not managed), soil pH (Table 1), gravimetric water content (data not shown), nitrate concentration (Table 1), ammonium concentration (Table 1; personal communication I Schöning), total carbon content (personal communication I Schöning) and total nitrogen content (personal communication I Schöning)) was tested using the software Canoco (version 4.5, Microcomputer Power, Ithaca, NY, USA) (Ter Braak and Smilauer, 2002). Effect of each variable on the community structure based on *mxaF*, *mch*, *fae* and MPN data sets was tested by permutation test (9999 replicates, Monte–Carlo test) of the same software package. First and second axes of CCA results were visualized in ordination diagrams (CanoDraw, Microcomputer Power). As vegetation type was significantly correlated with all data sets, indicator genotypes were determined to affiliate vegetation type to defined genotypes. Indicator values for vegetation type were calculated using the software PC-ORD (version 4.01, MjM Software Design) (Dufrene and Legendre, 1997). Indicator values for defined OTUs that were between 57% and 100% (*P*-value <0.04; Monte–Carlo test (1000 permutations)) were regarded as to be significant. Correlation of single MPN data sets (that is, highest or average MPN values; Supplementary Table S2) with environmental parameters was tested by Spearman rank correlation (Excel-based tool XLSTAT 2012; Microsoft GmbH, Unterschleißheim, Germany).

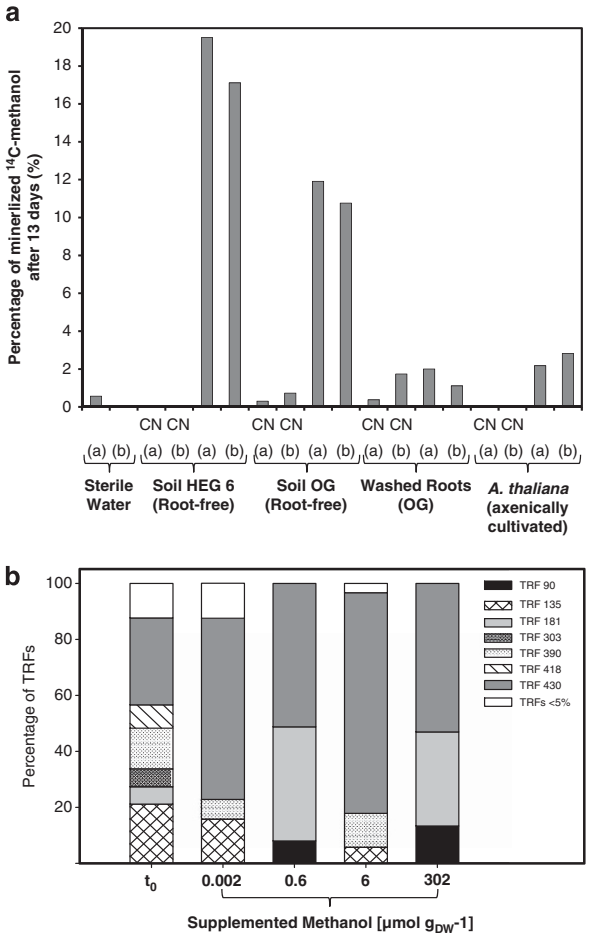

Figure 1 (**a**) Percentages of mineralized supplemented ^{14}C-methanol in soil and plant samples. CN, cyanide added (10 mM potassium cyanide) sterile water, abiotic control was autoclaved water. (**a**) and (**b**) Indices of experimental replicates. (**b**) Changes of relative frequencies of *mch* TRFs in slurries of soil HEG 6 after 13 days with different concentrations of methanol. Methanol dissimilation rates at 0.002, 0.6, 6.0 and 302.0 μmol methanol g_{soilDW}^{-1} ~0.0001, 0.027, 0.041, 1.185 μmol methanol g_{soilDW}^{-1} per day. Relative frequencies of TRFs shown at t_0 are means of four experimental replicates, and relative frequencies of TRFs at different concentrations after 13 days are means of TRF patterns of two experimental replicates.

Results

Methanol dissimilation and associated taxa in grassland soils

All experimental replicates of all three grassland soils that were tested for their capability to oxidize low concentrations (putatively ambient) of methanol to CO_2 (that is, HEG 6, OG and FG) exhibited similar dissimilation rates when methanol was provided at 74 nmol methanol g_{soilDW}^{-1} (that is, concentration corresponds to 10 μM in slurries). Observed half-saturation constants and maximal velocities were

Table 3 Effect of genotype composition of *mxaF*, *mch* and *fae*, and MPN data on environmental parameters by permutation tests

Data type	*Environmental parameter*[a]	P-*value*	*Trace value*
mxaF	Vegetation type	0.0007	0.275
	pH	0.0298	0.198
mch	Vegetation type	0.0001	0.322
	pH	0.0005	0.361
fae	Vegetation type	0.0018	0.433
	pH	0.0005	0.470
	Nitrate concentration	0.0390	0.341
MPN	pH	0.0069	0.109
	Ammonium concentration	0.0267	0.095

Abbreviation: MPN, most probable number.
[a]Only significantly correlated environmental parameters are presented and used for subsequent statistical analyses (Figures 2 and 3). The full set of tested environmental factors is available in online supplementary information (Supplementary Table S4).

different between the soils FG and HEG 6 (Table 2; Supplementary Figure S2). These kinetic parameters might have been affected by diffusion limitation during the incubation. Specific affinities, which are independent of diffusion limitation, were similar (Table 2). Hence, methanol-oxidizing enzymes with similar substrate affinities were active in both soils. Highest capacities of aerobic methanol dissimilation occurred in soil that was associated with roots (Figure 1a). Root material and micro-organism-free plant material of a typical grassland species (*A. thaliana*) displayed minimal activity (Figure 1a).

The TRF 430 bp was enriched at all supplemental methanol concentrations, and TRF 180 bp was enriched at concentrations greater than 600 nmol methanol g_{soilDW}^{-1} (Figure 1b). TRFs 430 and 180 bp were assigned to two novel genotypes (Grassland Soil Clusters (GSC) 1 and 2) within the *Alphaproteobacteria* (Supplementary Figure S3) based on comparison of measured TRFs with hypothetic TRFs, but were only distantly related to known genotypes of *mch* (30.9% distance on amino-acid level to *Starkeya novella* DSM 506 and 28.9% to *Methylocella palustris* BL2 for GSC1 and 2, respectively).

Correlations between genotype composition and environmental parameters

Genotype composition was significantly affected by soil pH, vegetation type (forest and grassland) and nitrate concentrations (Table 3; Figures 2a–c). Two *mxaF* genotypes, one *mch* genotype and one *fae* genotype were indicator genotypes for forest, while one *mxaF*, three *mch* and two *fae* genotypes were indicator genotypes for grassland (Table 4). The parameter vegetation type integrates numerous different environmental parameters, such as pH, gravimetric water content, ammonium and nitrate concentration. The latter three may represent a proxy for soil water regime and inorganic nitrogen availability. However, only nitrate concentration was significantly correlated with *fae* gene composition (Table 3).

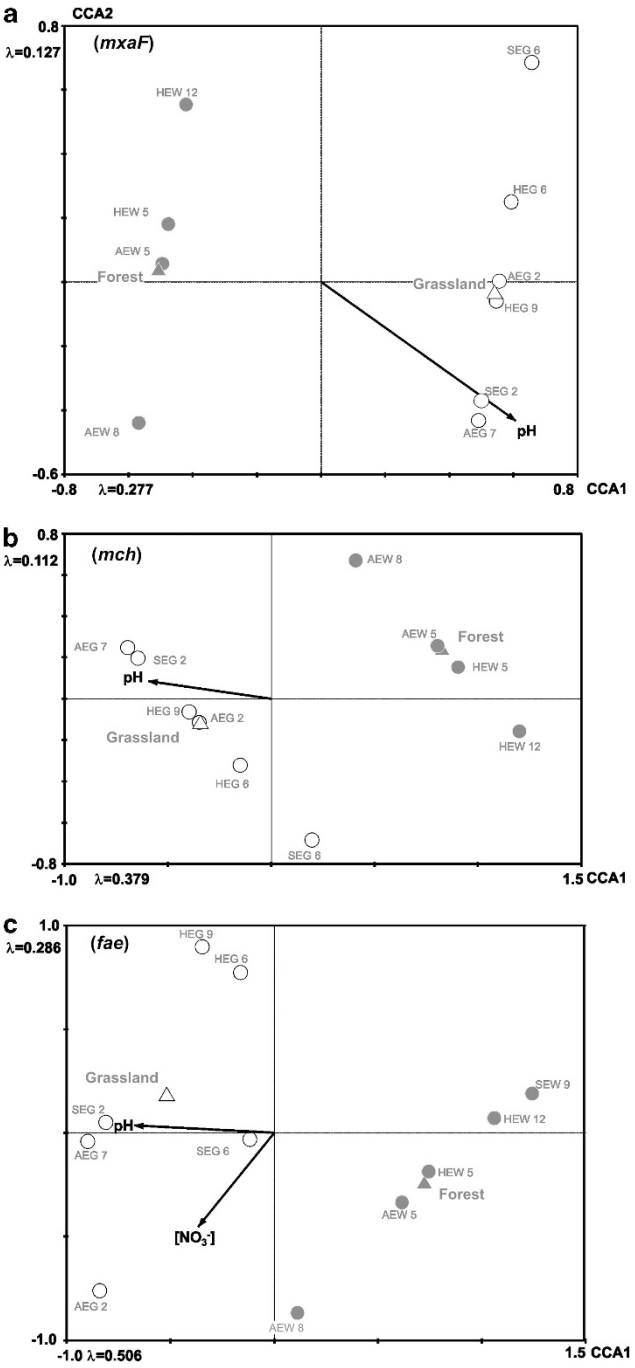

Figure 2 Effect of soil pH and vegetation type on *mxaF*, *mch* (**a**, **b**) genotype composition, and effect of soil pH, nitrate concentration, vegetation type on genotype composition of *fae* (**c**). Soil pH and nitrate concentration were used as vectors in CCA plots as they were significantly correlated to the respective genotype composition (Table 3). No data were available for *mxaF* of forest soil SEW 9. Thus, the data point of SEW 9 is missing in panel a. Closed circles, data from forest soils. Open circles, data from grassland soils. pH, soil pH; $[NO_3^-]$, nitrate concentration; λ, eigen value.

Table 4 Indicator genotypes for vegetation types

Gene	*GT*	*Vegetation type*	*IV (%)*	P
mxaF	6	Forest	99.0	0.023
	15	Forest	94.7	0.015
	2	Grassland	56.9	0.006
mch	185	Grassland	100	0.006
	184	Forest	90	0.025
	279	Grassland	83.3	0.042
	281	Grassland	83.3	0.047
fae	1233	Forest	100	0.002
	1206	Grassland	97.7	0.004
	1222	Grassland	83.3	0.014

Abbreviations: GT, genotype; *P*, value of significance; IV, indicator value.

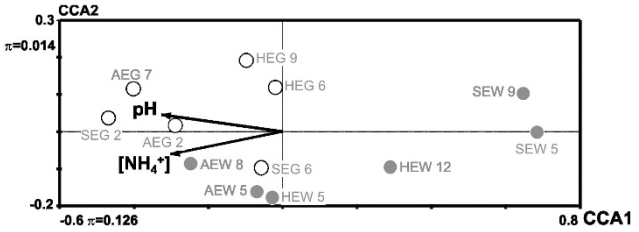

Figure 3 Effect of soil pH and ammonium concentration on MPNs (combined values of nine treatments) of methanol-grown methylotrophs. Soil pH and ammonium concentration were used as vectors in CCA plots as they were significantly correlated to the MPN data (Table 3). Closed circles, data from forests. Open circles, data from grassland soils. λ, eigen value.

Abundances of cultivated methylotrophs correlated with environmental parameters

Abundances of methylotrophs as determined by MPN analysis ranged from 1×10^6 cells g_{soilDW}^{-1} to 3×10^8 cells g_{soilDW}^{-1}. Highest cell numbers were determined for AEG 2 (that is, $(3 \pm 7) \times 10^8$ cells g_{soilDW}^{-1}), and lowest for HEG 6 (that is, $(1 \pm 2) \times 10^6$ cells g_{soilDW}^{-1}) (Supplementary Table S2). A correlation between highest or average MPNs of soils with any of the tested parameters was not significant (that is, tested by Spearman rank correlation). However, combined data sets of abundance data (that is, per soil MPNs of nine different cultivation treatments, Supplementary Table S2) significantly correlated with soil pH and ammonium concentration, but not with vegetation type based on Monte–Carlo tests. However, vegetation type was a determinative environmental parameter for genotype community composition (Table 3), and the combined set of abundances of forest soils were largely separated from those of grassland soils (Figure 3).

Genotype diversity of mxaF, mch and fae

In total, 32 898, 26 503 and 17 500 reads that started with the forward primers of *mxaF*, *mch* and *fae* amplicons were retrieved from 11 top soils, respectively (Table 1; Supplementary Table S5). 31, 70 and 63 genotypes were detected for *mxaF*, *mch* and *fae*, respectively. Estimated maximal numbers of genotypes were 31, 76 and 72 for *mxaF*, *mch* and *fae*, respectively (Supplementary Table S5). Genotype numbers were slightly reduced after rarefaction of data sets (Supplementary Table S5). Estimated numbers of *mch* and *fae* genotypes were greater than those of *mxaF* (Supplementary Table S5). The numbers of *mch*- and *fae*-like genes were greater suggesting a broader diversity of detected methylotroph taxa. *mch*- and *fae*-like genes also occur in non-methylotrophs (Chistoserdova *et al*., 2009; Chistoserdova, 2011). Thus, the high diversity of novel *mch* and *fae* genotypes may be additionally caused by codetected non-methylotrophs.

The diversity of detected genotypes was unevenly distributed and dominated by two genotypes of *mxaF*, namely OTUs 2 (78% of all sequences) and 6 (13%). OTU 2 affiliated with *Methylobacterium* (Figure 4). Most abundant *mch* OTUs were 178 (46%), 201 (15%), 186 (6%) and 185 (5%). *fae* genotypes were more even distributed (Supplementary Table S5), which was reflected by a larger number of genotypes with a relative abundance above 1%, that is, those were 1193 (53%), 1190 (12%), 1233 (10%), 1209 (3%), 1212 (4%), 1222 (3%), 1205 (3%) and 1206 (2%).

Methylobacterium-affiliated *mxaF* genotypes were predominant in all soils and exhibited relative abundances of >59% in each soil (data not shown). A substantial number of low-abundant genotypes (that is, OTUs 15, 16, 17, 18, 19, 21, 22, 23, 24, 25, 26 and 27) were not closely affiliated with genotypes of known methylotrophic species. These low-abundant genotypes affiliated with genome-derived genotypes of bacterial strains, of which methylotrophy is not known to date (for example, with *mxaF*' of *Rhizobiales*) (Figure 4).

Dominant *mch* genotypes and *mxaF* genotypes affiliated with *Alphaproteobacteria* but with different genera, that is, *mch* genotypes, were distantly related with *Granulibacter* and *Methylocella* (OTU 201), and *Starkeya* (OTUs 178, 185 and 186) (Supplementary Figure S3). The two *mch* genotypes (GSC 1and 2) that responded to low methanol concentrations in soil HEG 6 (Figure 1b) were closely related on amino-acid level with the two most abundant *mch* genotypes (OTUs 178 and 201) (Supplementary Figure S3). Some *Alphaproteobacteria*-affiliated *mch* genoytpes were only distantly related to *mch* genes of known isolates (Supplementary Figure S3) suggesting that numerous unknown *Alphaproteobacteria* harbor *mch* genes.

All detected *fae* genotypes belonged to *fae* genes *sensu stricto* as classified in a previous study (Chistoserdova, 2011), and were mainly affiliated with those of *Alpha*, *Beta*- and *Gammaproteobacteria* (Supplementary Figure S4). The most abundant genotypes (OTUs 1193, 1190 and 1233, which collectively constituted 75% of all *fae* reads) were affiliated with members of *Alphaproteobacteria* (Supplementary Figure S4). Owing to the low number of known reference sequences and low

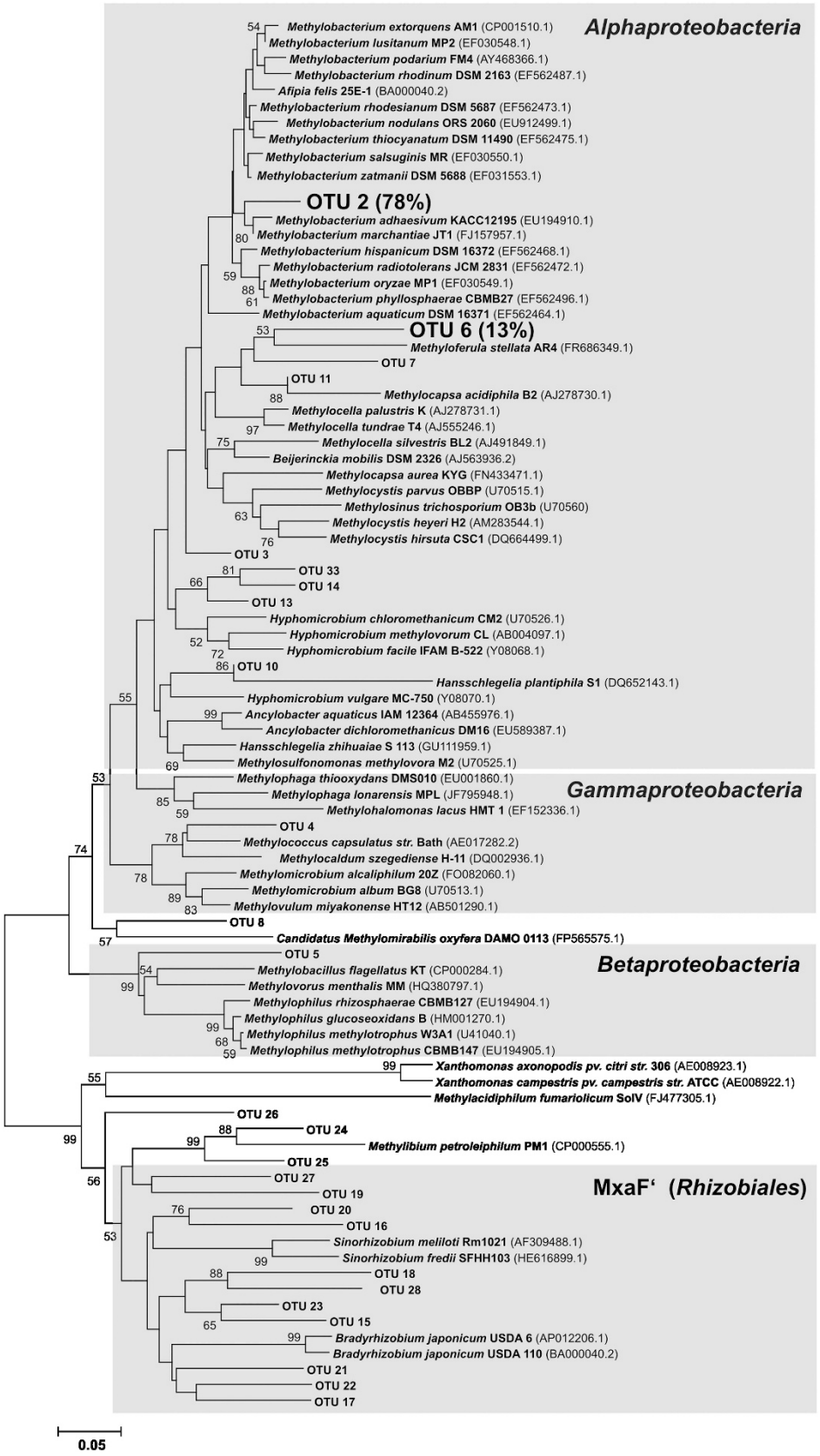

Figure 4 MxaF protein tree of detected genotypes. Bold genotypes, genotypes with relative abundances >1%, i.e., relative frequencies are given in parentheses. Numbers at nodes, bootstrap values from 1000 replicated trees, those >50% are shown. Scale bar represents an evolutionary distance of 5%. OTU, *mxaF* genoytpes of rarefied data sets.

similarity to known ones, it was difficult to determine if detected OTUs represented known or novel genera. The most closely related isolates for many *fae* OTUs were well-known methylotrophs, such as *Hyphomicrobium*, *Methylobacterium*, *Methylibium* and *Methylotenera*, but several OTUs were closely related to *Burkholderia* species, which are not known to be capable of methylotrophy (Kolb, 2009a).

A substantial number of novel genotypes of *mch* and *fae* were not affiliated with isolated species (Supplementary Figures S3 and S4). As the diversity of known *mch*- and *fae*-like genes is not restricted to methylotrophs (Chistoserdova, 2011), it is likely that the detected deep-branching *mch* and *fae* genotypes does not exclusively represented aerobic methylotrophs.

Discussion

Methanol-utilizing methylotrophs have been known since the nineteenth century, and have been repeatedly isolated from soils worldwide (Loew, 1892; Lidstrom, 2006; Chistoserdova *et al.*, 2009; Kolb, 2009a). Methanol-utilizing methylotrophs can be metabolically stimulated by adding millimolar concentrations of methanol to aerated and flooded soil (Radajewski *et al.*, 2002; Lueders *et al.*, 2004). However, the methylotrophs responsible for *in situ* methanol consumption are unresolved. Information on ranges of methanol concentrations in soil and on 'hot spots' of methanol production are not available. Concentrations down to nanomoles per gram dry weight of soil (that is, corresponds to about 0.1 μM in slurries) were oxidized in the current study suggesting that methylotrophs have methanol-oxidizing enzymes with micro to nanomolar half-saturation constants that could oxidize such low methanol concentrations. Based on maximal atmospheric methanol concentrations (10 p.p.b.) (Galbally and Kirstine, 2002; Jacob *et al.*, 2005), it can be estimated that diffusion into soil yield concentrations of several nanomoles of methanol per gram dry weight of soil (that is, $\sim$20 nmol methanol g^{-1}_{soilDW}). Thus, the observed capacity of soil microorganisms to oxidize concentrations at nanomoles methanol g^{-1}_{soilDW} may explain the capacity of aerated soils to consume atmospheric methanol (Karl *et al.*, 2005; Schade *et al.*, 2011). Observed minimal methanol oxidation rates were even below 20 nmol methanol g^{-1}_{soilDW}. It can be speculated that unknown *Alphaproteobacteria* (that is, as suggested by responding *mch* genotypes) might have high-affinity methanol dehydrogenases that enable them to utilize atmosphere-derived methanol.

Methanol oxidation by methylotrophs in grassland soils FG, HEG 6 and OG

Two novel *mch* genotypes (GSC 1 and 2) responded to concentrations of methanol below 1 μmol g^{-1}_{soilDW}. GSC 1 and 2 were distantly related to *mch* genotypes of known methylotrophs, and grouped with those of *Alphaproteobacteria*. Although amplification of *mch* failed in several samples of the kinetic experiments that were conducted with the soils FG and HEG 6, gene marker-based assessment of methylotroph community structures in the remaining 11 aerated soils revealed that *Alphaproteobacteria* represented the most frequently detected phylum. The *mch* data set suggested that GSC 1- and 2-like genotypes were frequently detected putative methanol utilizers in both temperate grasslands and forests.

Substrate affinities of active methylotrophs in grassland soils FG and HEG 6 were in the upper range of values of purified MDH (Arfman *et al.*, 1989; Hektor *et al.*, 2002), and higher than those of a previous environmental study that addresses ocean surface water communities (Dixon *et al.*, 2011). Accordingly, the specific affinity values a^s_o were lower, and v_{max} were higher than the solely known values that have been obtained from the environment (a^s_o: 0.12 to 0.96 d^{-1}; v_{max}: up to 24 nmol d^{-1} l^{-1}) (Dixon *et al.*, 2011). The lowest measured K_m values (3 μmol l^{-1}) of MDH are known of the Gram-negative soil methylotroph *Hyphomicrobium denitrificans* (Nojiri *et al.*, 2006), whereas K_m values of MDH of other soil methylotrophs can be much higher (that is, *Bacillus methanolicus,* $K_m > 200$ μmol l^{-1}) (Arfman *et al.*, 1989; Hektor *et al.*, 2002). *H. denitrificans* has a PQQ MDH (encoded by MxaFI) that has been intensively studied in the model methylotroph *M. extorquens* AM1 (Chistoserdova *et al.*, 2009). PQQ MDH also occurs in many other Gram-negative methylotrophs (Nunn *et al.*, 1989; Schmidt *et al.*, 2010; Chistoserdova, 2011). Utilization of micro molar concentrations is known from *M. extorquens* AM1, and the marine methylotrophic strain HTCC2181 (*Betaproteobacteria*) (Giovannoni *et al.*, 2008; Halsey *et al.*, 2012). Strain HTCC2118 assimilates 10 μmol l^{-1} methanol, which is substantially above observed minimal concentrations (0.002 μmol g^{-1}_{soilDW}) at which methanol dissimilation was measured in the present study. Hence, it is likely that various soil methylotrophs have the capability of dis- and assimilation of concentrations above several hundred nanomoles of methanol per gram dry weight of soil. If those can also be active at minimal concentrations observed in the current study remains speculative.

Many soil methylotrophs are facultatively methylotrophic, that is, can utilize multicarbon compounds (Loew, 1892; Lidstrom, 2006; Chistoserdova *et al.*, 2009; Kolb, 2009a). Moreover, it has been demonstrated that methylotrophs that are restricted to methanol as carbon source exhibit increased methanol assimilation rates when they codissimilate alternative monocarbon compounds (Halsey *et al.*, 2012). Thus, it is likely that methanol utilizers in aerated soils are not restricted to ambient methanol utilization and occupy different ecological niches in regard to the alternative substrates they can metabolize (Kolb, 2009a).

High methanol dissimilation capacity in root-associated grasslands soil

OG and HEG 6 root-associated soils exhibited the highest methanol oxidation activities, indicating that microorganisms in root-associated soil were mainly responsible for the capacity of soils to oxidize methanol. Root-free soil samples were gained by a mild washing procedure with sterile water suggesting that soil methylotrophs of grasslands are not necessarily tightly associated with the plant surface as are phyllosphere methylotrophs (for example, Delmotte *et al.*, 2009). Roots constitute likely a source of methanol, and the minimal capacity of roots to oxidize methanol (Figure 1a) suggests that roots are not net sinks for methanol in grassland soils.

Environmental parameters that shape methylotrophic communities in aerated soils

All three gene marker data sets and the combined MPN analyses were significantly correlated with soil pH and, in case of gene data sets, also by vegetation type suggesting that the observed influences of environmental parameters are largely method independent. Vegetation type and soil pH are site parameters that are stable over several years (Fischer *et al.*, 2010). The more dynamic site parameters (that is, water content, ammonium and nitrate concentration) did not significantly affect all of the data set patterns. This lack of general correlation does not necessarily mean that such dynamic parameters were irrelevant, but it is more likely that a seasonal data set of these parameters and methylotroph communities would have led to more conclusive results. Moreover, *fae* genotype composition was significantly affected by nitrate and MPN data set by ammonium concentration indicating that nitrogen availability was also a key parameter for soil methylotrophs, which is in accordance with knowledge of physiologies of methylotrophs that can assimilate dinitrogen gas or nitrate and ammonium as sources of nitrogen (Kolb, 2009a, and references therein).

Soil pH is a factor that can be correlated with soil prokaryotic community structure (Fierer and Jackson, 2006). A correlation between soil pH and aerobic methanotroph genotypes is also likely (Kolb, 2009b). Known methylotrophic species cover the full range of pH optima, that is, from alkalophilic to acidophilic species (Lidstrom, 2006; Kolb, 2009a, 2009b and references therein). Plant species composition can also be correlated with the overall community structure of soil bacteria (Berg and Smalla, 2009). Methylotroph plant interactions can be very specific (for example, root nodule formation) or can be less stringent when soil methylotrophs modulate growth of their host plants (for example, Sy *et al.*, 2001; Fedorov *et al.*, 2011). In the case of methane-oxidizing methylotrophs, the presence of trees correlates with certain methanotrophic genotypes (Kolb, 2009b; Degelmann *et al.*, 2010). Thus, a growing body of evidence supports the hypothesis that community structure of soil methylotrophs in temperate grassland and forest soils is influenced by plant diversity and soil pH.

Diversity of soil methylotrophs

Alphaproteobacterial methylotrophs dominated the soil communities. Knowledge of the *in situ* diversity of methanol-utilizing methylotrophs in soils is limited despite the fact that many soil-derived methanol-utilizing methylotrophs have been isolated or detected by molecular analyses by employing high and likely not *in situ*-relevant methanol concentrations (Radajewski *et al.*, 2002; Lueders *et al.*, 2004; Lidstrom, 2006; Kolb, 2009a). Methanol-utilizing *Burkholderiaceae*, *Beijerinkiaceae* and *Bradyrhizobiacea* occur in aerated soils (Radajewski *et al.*, 2002). These taxa as well as a larger diversity of known and unknown soil methylotrophs were detected in the current study. The gene survey revealed a large number of novel genotypes within the *Proteobacteria. Actinobacteria* and *Flavobacteria* were frequently isolated by cultivation with methanol-supplemented mineral media (Supplementary Figure S5); that these taxa were not revealed by the gene survey suggests that targeting methylotrophs by cultivation may identify additional taxa in the overall diversity of methanol-utilizing microorganisms.

The dominance of *Methylobacterium* in phyllosphere communities (Holland *et al.*, 2002; Delmotte *et al.*, 2009; Wellner *et al.*, 2011; Mizuno *et al.*, 2012) is coincident with the finding that *Methylobacterium*-affiliated genotypes were predominant in the *mxaF* data sets of investigated soils. *Methylobacterium*-affiliated *mxaF* genoytpes were statistically indicative for grassland vegetation. The phyllosphere is inoculated by methylotroph community of soil during plant growth (Romanovskaya *et al.*, 2001), which is supported by the finding that *Methylobacterium* species were important soil methylotrophs. Various *mxaF* and *mch* genotypes affiliated with genotypes of *Rhizobiales* (Figure 4; Supplementary Figure S3) that are not known to be capable of methylotrophy (Lidstrom, 2006; Kolb, 2009a). Similar genotypes were also detected in a forest soil by methanol-dependent stable isotope probing (Radajewski *et al.*, 2002).

Some *fae* genotypes were affiliated with aerobic members of *Planctomycetales* and *Leptothrix* (Supplementary Figure S4). However, it is not likely that these taxa are capable of aerobic methylotrophy (Lidstrom, 2006; Chistoserdova *et al.*, 2009; Kolb, 2009a). Thus, the *fae* analysis likely detected some non-methylotrophs and biased the statistical analyses. *mch* and *fae* genotype analyses also suggest that hitherto unkown methylotrophic taxa were present.

A limitation of gene markers currently available for the detection of methylotrophs is that they are

biased towards Gram-negative methylotrophs. *mch* and *fae* only occur in those methylotrophs that employ the H_4MPT-dependent formaldehyde oxidation pathway (Kalyuzhnaya *et al*., 2004; Kalyuzhnaya and Chistoserdova, 2005; Chistoserdova, 2011). *mxaFI* genes have been found in some *Actinobacteria* and methylotrophy are widely distributed in this taxon (Anesti *et al*., 2004; Hung *et al*., 2011). Biochemically and evolutionary distinct methanol oxidoreductases of Gram positives cannot be detected with currently available primers (for example, those of *B. methanolicus*; Hektor *et al*., 2002; Naerdal *et al*., 2011). Thus, future efforts are needed to improve the assessment of Gram-positive methylotrophs.

Conclusions

This study demonstrated that atmospheric methanol concentrations can be dissimilated by methylotrophs in temperate soils. It remains speculative if methylotrophic methanol consumption in soil can explain the large gap between theoretical production and observed emission into the atmosphere. *Alphaproteobacteria*, especially those that are represented by *mch* genotypes GSC 1 and 2, may be capable of utilization of atmospheric methanol. In addition to *Methylobacterium,* other taxa may be important methanol consumers in temperate ecosystems. The correlation of vegetation type and the community structure of methylotrophs suggests that interactions between plants and methylotrophs are important to methylotroph diversity in aerated soil. The flux of methanol in temperate grassland and forest ecosystems is likely linked to the broad diversity of soil methylotrophs, and further resolution of ecological niches and physiological properties of such methylotrophs is warranted.

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