The ISME Journal (2009) 3, 606–617

www.nature.com/ismej

ORIGINAL ARTICLE

A reanalysis of phospholipid fatty acids as ecological biomarkers for methanotrophic bacteria

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Aerobic methane-oxidizing bacteria (MB) are the primary terrestrial sinks for the greenhouse gas methane. A distinct characteristic of MB is the presence of specific phospholipid ester-linked fatty acids (PLFA) in their membranes that differentiate them from each other and also from all other organisms. These distinct PLFA patterns facilitate microbial ecology studies. For example, the assimilation of C from methane into PLFA can be traced in environmental samples using stable isotope (^{13}C) probing (SIP), which links the activity of MB to their community composition *in situ.* However, the phylogenetic resolution of this method is low because of a lack of PLFA profiles from cultured MB species. In this study, PLFA profiles of 22 alphaproteobacterial (type II) MB were analysed after growth on methane, methanol or both substrates together. Growth on different substrates did not affect the PLFA profiles of the investigated strains. A number of *Methylocystis* strains contained novel C18:2 fatty acids (ω7c,12c and ω6c,12c) that can be used as diagnostic biomarkers. The detection of these novel PLFA, combined with the analyses of multiple type II strains, increased the phylogenetic resolution of PLFA analysis substantially. Multivariate analysis of the expanded MB PLFA database identified species groups that closely reflected phylogenies based on 16S rRNA and *pmoA* gene sequences. The PLFA database therefore provides a robust framework for linking identity to activity in MB communities with a higher resolution than was previously possible.

The ISME Journal (2009) **3,** 606–617; doi:10.1038/ismej.2009.6; published online 5 February 2009
Subject Category: microbial ecology and fuctional diversity of natural habitats
Keywords: methanotrophs; methane; PLFA; fatty acids; biomarkers

Introduction

Methane is the second most important greenhouse gas after carbon dioxide (Solomon *et al.*, 2007). As the methane concentration in the atmosphere has more than doubled in the postindustrial era (Solomon *et al.*, 2007), much research effort has been expended to identify sources and sinks of methane, and the organisms involved. Usually, the balance between the production of methane by methanogenic archaea under anoxic conditions and the consumption of methane by methanotrophic bacteria (MB) under oxic conditions determines whether a particular environment acts as a source or a sink for atmospheric methane. Methane can also be oxidized anaerobically (Hinrichs *et al.*, 1999; Boetius *et al.*, 2000). However, the microbes involved in the anaerobic process are not the subjects of this study.

Submerged wetland soils (for example, swamps, bogs, rice paddies) are the most important source of atmospheric methane whereas non-flooded upland soils (for example, forests, grassland, arable) are the only net biological sink for atmospheric methane (LeMer and Roger, 2001). In both wetland and upland soils, obligate aerobic MB use molecular oxygen to oxidize methane to CO_2 and cell carbon (Hanson and Hanson, 1996; Conrad, 2007; Dunfield, 2007). In wetland soils MB are active in the surface

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Received 11 November 2008; revised 8 January 2009; accepted 8 January 2009; published online 5 February 2009

soil layers, in the rhizosphere of oxygen-releasing plants (Frenzel, 2000) and even inside plant tissues (Raghoebarsing *et al.*, 2005), thereby reducing the potential amount of methane emitted by up to 90% (Solomon *et al.*, 2007). In dry upland soils their activity accounts for approximately 6% of the global sink strength of atmospheric methane.

Taxonomically, all known MB belong to two phyla: *Proteobacteria* and *Verrucomicrobia*. The latter have only recently been discovered (Dunfield *et al.*, 2007; Pol *et al.*, 2007; Islam *et al.*, 2008), and are not yet taxonomically validated. The proteobacteria have been studied for much longer, and there are now 16 recognized genera within the *Alphaproteobacteria* and *Gammaproteobacteria* classes of this phylum. The *Alphaproteobacteria* MB include two distinct families, *Methylocystaceae* (genera *Methylosinus* and *Methylocystis*) and *Beijerinckiaceae* (genera *Methylocella* and *Methylocapsa*). The *Gammaproteobacteria* MB are all presently classified into the family *Methylococcaceae* (*Crenotrichaceae* is a phylogenetic subset of *Methylococcaceae*). Traditionally, the use of valid taxonomic names above the level of genus for MB has been eschewed in favour of a simple and artificial division into two groups: type I and type II. This division was originally postulated on the basis of phylogeny (*Gamma-* versus *Alphaproteobacteria*), carbon fixation pathway (ribulose monophosphate versus serine pathway), arrangement of internal membrane stacks (perpendicular versus parallel to the cell envelope), and phospholipid fatty acid profiles (see below; Hanson and Hanson, 1996; Conrad, 2007). Although the biochemical and morphological generalizations have proven useful in some studies, recent discoveries of new species have shown that these generalizations are not universal, so the terms type I and type II are now generally used in a purely taxonomic sense as synonyms for *Gammaproteobacteria* and *Alphaproteobacteria*, respectively.

The proteobacterial MB possess distinct patterns of phospholipid ester-linked fatty acids (PLFA) that differentiate them from each other. The type I MB contain mainly 14C and 16C PLFA, whereas the type II contain mainly 18C PLFA. This difference is practical for distinguishing methane consumption by type I versus type II species using isotope labelling. In addition, MB possess signature fatty acids that are not found in any other known microorganism (type I: C16:1ω8c and C16:1ω5t versus type II: C18:1ω8c) and are therefore especially valuable biomarkers (Nichols *et al.*, 1985; Bowman *et al.*, 1991, 1993). The specificity of these signatures, combined with PLFA being structural components of cell membranes, enables the use of PLFA to estimate abundance of these bacteria in various environments (Sundh *et al.*, 1995; Borjesson *et al.*, 1998; Costello *et al.*, 2002; Mohanty *et al.*, 2006).

Tracing the incorporation of methane-C into PLFA using stable isotope (^{13}C) or radioisotope (^{14}C) labelling (Roslev *et al.*, 1998; Bodelier *et al.*, 2000; Henckel *et al.*, 2000) enables researchers to link the biogeochemical function of these bacteria with their phylogeny and community composition. Stable isotope probing of PLFA (SIP-PLFA) has extensively been applied to assess the active methane consumers in soil or sediment environments (for example, Knief *et al.*, 2003; Evershed *et al.*, 2006; Chen *et al.*, 2008; Shrestha *et al.*, 2008). SIP-PLFA of environmental samples using $^{13}CH_4$ yields a pattern of ^{13}C-labelled environmental PLFA that can be compared to PLFA profiles of known MB cultures to infer the identities of environmentally active MB. For example, it was shown that the active MB in forest soils varied depending on soil pH: a type I species dominated in neutral soils whereas a type II species dominated in acidic soils (Knief *et al.*, 2003). Shifts between type II and type I species also occur with changing methane supply in soils (Henckel *et al.*, 2000; Knief *et al.*, 2006) or with application of nitrogen fertilizer (Mohanty *et al.*, 2006). However, virtually all SIP-PLFA studies performed to date have not yielded a level of resolution beyond the level of type I versus type II MB (that is, the classes *Gammaproteobacteria* versus *Alphaproteobacteria*). A major reason for this limitation is that a collective PLFA database is not available and that the number of profiles available for type II MB is limited and lacks resolution. MB classification on a PLFA basis is still based primarily on studies made between 1985 and 1993 (Nichols *et al.*, 1985; Bowman *et al.*, 1991, 1993). The availability of a large number of new type II strains (Heyer *et al.*, 2002) initiated this study. The objective was to collect all currently available MB PLFA profiles and expand the existing type II PLFA database by analysing new strains, to obtain a comprehensive framework for SIP-PLFA studies. To meet this objective, 22 type II strains were grown on methane, methanol or methane + methanol, and PLFA profiles were then obtained. Multivariate analyses of the profiles yielded a classification framework that closely reflected DNA-based phylogeny.

Methods

Organisms used in this study

The following type II strains were analysed for PLFA analyses: *Methylocystis* sp. SC2, sp. M31, sp. LR1, sp. 62/12, sp. KS33, sp. KS8a, sp. IMET10484, sp. Pi5, sp. KS31, sp. F10v2a, *Methylocystis echinoides* IMET10491, *Methylosinus sporium* KS17, 8, *Methylosinus trichosporium* KS21, SM6, KS24b, SC10, O19. All these strains have been described (Heyer *et al.*, 2002). The strains *Methylocystis* sp. H4 and H17 and *M. sporium* H7 were isolated from a river floodplain soil as described by Bodelier *et al.* (2005). *Methylocystis* sp. Rice86 was isolated from an Italian rice soil (Bodelier *et al.*, 2000) using the procedure reported (Bodelier *et al.*, 2005).

Growth conditions of bacteria

Methylocystis sp. SC2, sp. M31, sp. LR1, sp. 62/12, sp. KS33, sp. KS8a, sp. IMET10491, sp. IMET10484, sp. Pi5, sp. KS31, sp. F10v2a, *M. sporium* KS17, 8, *M. trichosporium* KS21, SM6, KS24b, SC10, O19 were grown in 1-l erlenmeyer flasks containing 400 ml of M10–NMS medium (Heyer *et al.*, 2002) on a shaker (120 r.p.m.) at 25 °C. Methane-grown cultures received 10% (v/v) CH_4 in the headspace whereas 2 ml (0.5%) methanol was added to cultures grown on methanol.

The strains *Methylocystis* sp. H4 and H17 and *M. sporium* H7 were grown in 150 ml of M10–NMS medium (Heyer *et al.*, 2002) with a headspace of 20% CH_4 in air. The cultures were harvested by centrifugation (21 875 *g*, 30 min, 15 °C). Pellets were freeze dried and stored at −20 °C. For structure analyses of the novel C18:2 PLFA,*Methylocystis* sp. Rice 86 was grown in a 10-l glass vessel containing 5 l of M10 medium. The headspace consisted of 20% CH_4 in air. The headspace of the flasks was connected to a gas bag filled with air to prevent underpressure in the flasks following methane and oxygen consumption. In total 120 l of culture was harvested to obtain approximately 1.5 g of dry cell material.

PLFA analyses

Lipids were extracted from 2–6 mg of freeze-dried cell material using a modified Bligh and Dyer extraction procedure (Boschker *et al.*, 1998, 2001). The lipid extract was fractionated on silicic acid columns into different polarity classes by sequential elution with chloroform, acetone and methanol. The methanol fraction containing the PLFA was subjected to mild-alkaline methanolysis to yield fatty acid methyl esters (FAME). C12:0 and C19:0 authentic FAME standards (Sigma-Aldrich, St Louis, MO, USA; 100 µl of 0.1 mg ml^{-1} stock solution) were used for calculating retention indices and for quantification and were added before the methylation step of the fatty acids. C19:0 is used for quantification because of its absence in known MB. FAME concentrations were determined using a gas chromatograph (Thermo Finnagan TRACE GC) equipped with flame ionization detector (GC-FID) and a polar capillary column (SGE, BPX-70, 50 m × 0.32 mm × 0.25 µm) using the following oven conditions: initial temperature 50 °C for 1 min, then increasing to 130 °C by 40 °C min^{-1}, then increasing to 230 °C by 3 °C min^{-1}. Identification of FAMES was based on comparison of the retention times of FAMES with those of the authentic FAME standards and in selected cases by GC mass spectrometry (MS). Additional support for identification of FAMES for which no commercial standards are available was obtained using type-culture extracts (*Methylomonas methanica* S1 NCIMB 11130; *Methylomicrobium album* NCIMB 11123^T; *Methylobacter luteus* NCIMB 11914^T; *Methylocystis parvus* NCIMB 11129^T; *M. trichosporium* NCIMB 11131^T; *M. sporium* NCIMB 11126T) of which the PLFA profiles are known.

To determine double-bond position of monounsaturated PLFAs that could not be identified by retention index and GC-MS data, dimethyl disulphide (DMDS) derivitization was performed as described by Nichols *et al.* (1985). DMDS adducts were analysed on a Finnigan TRACE GC-MS system. DMDS adducts were separated using an Rtx-5MS (60 m × 0.32 mm × 0.25 µm) capillary column with the following oven conditions: initial temperature 80 °C for 1 min, then increasing to 160 °C by 40 °C min^{-1}, then increasing to 330 °C by 4 °C min^{-1}. MS operating parameters were: electron multiplier 375 V; source temperature 200 °C; transfer line temperature 250 °C; fullscan m/z 33–410. MS data were acquired and processed using the Finnigan Xcalibur software.

Identification of the unusual fatty acids in Methylocystis *spp.*

Approximately 1.5 g of freeze-dried material of *Methylocystis* sp. Rice 86 was subjected to base hydrolysis in 1 N KOH in methanol/water (96/4) under reflux for 1.5 h. After addition of water, the neutral lipids were extracted with dichloromethane (DCM) and, after acidification to pH 3, fatty acids were extracted with DCM. This yielded approximately 60 mg of fatty acids, which were subsequently methylated with BF_3/methanol at 60 °C for 20 min. A small aliquot was used to produce DMDS derivatives to locate the position of double bonds using the method of Vincenti (Vincenti *et al.*, 1987). To isolate the two unknown $C_{18:2}$ fatty acids (as methyl esters), the FAMEs were separated with argentation column chromatography (Ghosh *et al.*, 1972) using a column packed with SiO_2/5% $AgNO_3$ (80 × 12 mm; $V_0 = 6.5$ ml) and mixtures of 5, 10, 20 and 50 vol% of diethyl ether in hexane. This procedure was repeated on fractions containing the $C_{18:2}$ FAMEs using 7 and 8 vol% diethyl ether in hexane until two fractions (3.2 and 1.0 mg) were obtained that contained a $C_{18:2}$ FAME in an almost pure form (>80% as determined by GC and GC/MS). These fractions were dissolved in 0.75 ml C_6D_6 or $CDCl_3$ and analysed by ^1H and ^{13}C nuclear magnetic resonance (NMR) on a Bruker Avance 400 and Varian Unity Inova 500 spectrometers.

Statistical analyses

Differences between the total PLFA content of *Methylocystis* spp., *M. trichosporium* and *M. sporium* strains were tested using a *t*-test for independent samples using the Statistica software package version 8.0 (StatSoft Inc., Tulsa, OK, USA).

Multivariate analyses PLFA profiles

Bray–Curtis similarity matrices of the PLFA profiles (% of total PLFA) were square-root transformed to

even out the contribution of very rare and very dominant PLFAs, and analysed by cluster analyses as well as multidimensional scaling (MDS). Clustering was made using the group-average linking routine. The MDS analyses results in a two-dimensional plot where the distance between samples indicates the similarity of these samples relative to other samples in the plot. The accuracy of the two-dimensional representation in MDS is indicated by the 'stress' value (Kruskall's stress formula). Stress values <0.1 indicate a good ordination with no prospect of misleading interpretation. Stress values <0.2 still give a good two-dimensional representation, but other methods of representation should be used in parallel (for example, cluster analyses) to confirm the observed groupings. The ANOSIM procedure was used to test whether PLFA profiles differed between cultures grown on methane versus methanol. All clustering and MDS analyses were performed using the Primer-E software (Plymouth Marine Laboratory, Plymouth, UK). All theoretical aspects of the cluster and MDS analyses used are described by (Clarke and Warwick, 2001).

Results

PLFA analyses of type II MB

A large number (22) of different strains of type II MB belonging to the genera *Methylocystis* and *Methylosinus* were analysed for PLFAs. The PLFA profiles of these MB (in % of total PLFA) are listed in Supplementary Table 1a together with all MB profiles available in the literature (Supplementary Table 1b). In a number of *Methylocystis* strains previously unreported fatty acids were detected.

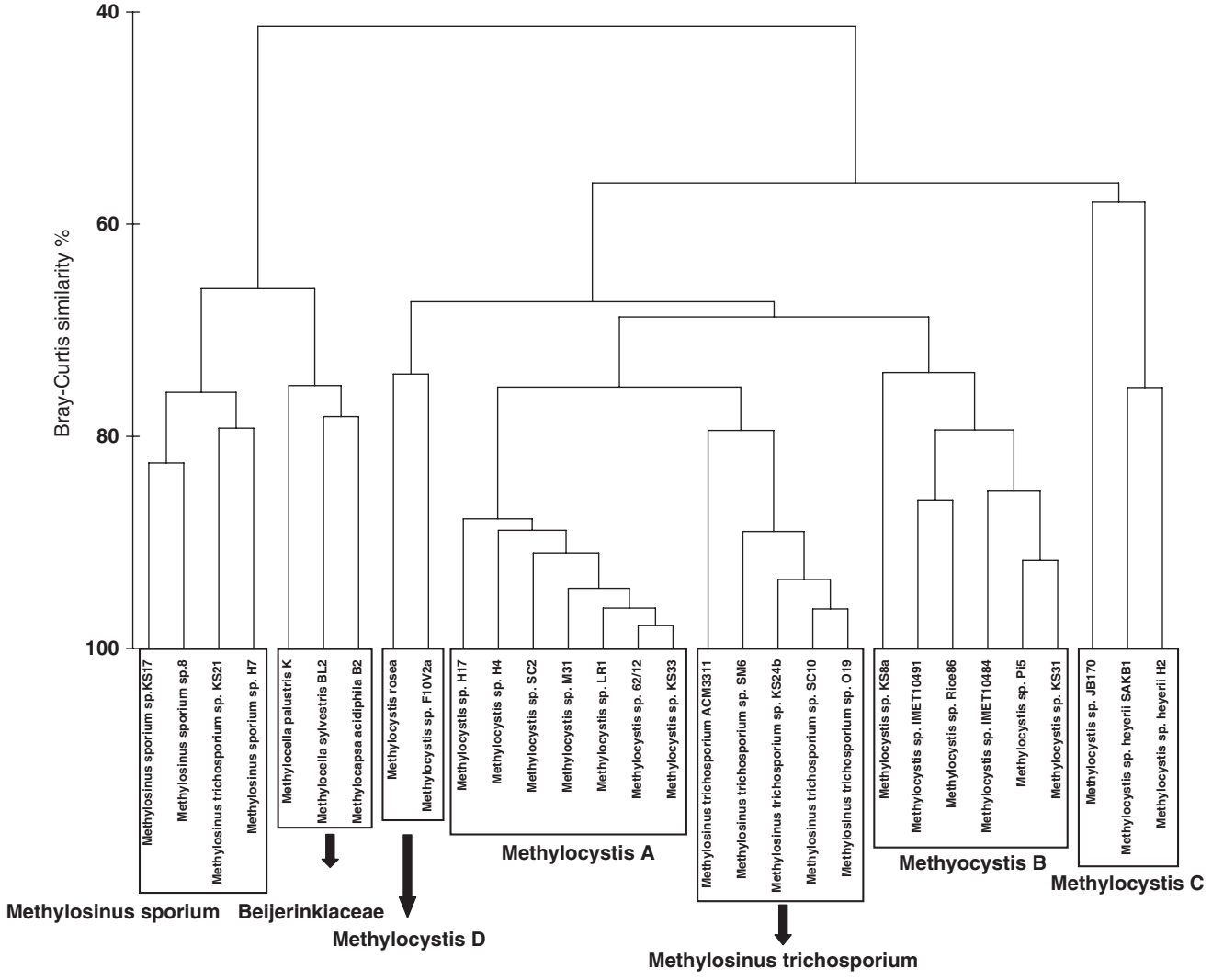

Figure 1 Multivariate statistical analyses of phospholipid ester-linked fatty acids (PLFA) profiles of type II methanotrophs grown on methane. The figure combines data obtained in this study with data from the literature (see also Supplementary Table 1a+b). The input of the cluster analyses were Bray–Curtis similarity matrices of square-root-transformed PLFA profiles (% of total PLFA content). The clustering was made using the Group Average linking Cluster analyses (UPGMA).

Identification of $C_{18:2}$ fatty acids in Methylocystis *sp. Rice 86*

To fully identify the two unknown fatty acids in the group B *Methylocystis*, a large batch of strain Rice 86 was grown and the fatty acids were isolated. This resulted in two fractions that contained the two fatty acids in a purity >80%. These fractions were studied by ^{1}H and ^{13}C NMR spectroscopy and a number of two-dimensional NMR techniques (COSY, HMBC and HSQC). The double bond positions of the fatty acids were elucidated by COSY experiments, which showed the double bonds to be at positions 7,12 (major isomer) and 6,12 (minor isomer) (Supplementary Table 2). These assignments of the double bond positions were confirmed by GC/MS analysis of the DMDS derivatives (Vincenti *et al*., 1987). The stereochemistry of the double bonds was established to be *cis*. This was established by determining the coupling constants of the olefinic protons in ^{1}H NMR experiments in which the allylic protons were decoupled by irradiating the signal at 1.95–2.15 p.p.m. The estimated ^{3}J (H,H) is 11 Hz, indicative for *cis* double bonds. This was confirmed by the chemical shifts of the allylic carbon atoms at approximately 27 p.p.m. (Supplementary Table 2). The chemical shifts of allylic carbon atoms of unsaturated fatty acids with *cis* and *trans* double bonds typically differ by approximately 6 p.p.m. (Gunstone *et al*., 1977). To fully confirm this, NMR measurements with the two $C_{18:2}$ FAME were also performed in $CDCl_3$ (Supplementary Table 2) and these data match those reported in the literature (Gunstone *et al*., 1977; Kulkarni *et al*., 1997).

Multivariate analyses of type II PLFA profiles

The PLFA profiles analysed in this study together with all available published profiles of other alphaproteobacterial methanotrophs were transformed into Bray–Curtis similarity matrices and subjected to multivariate analyses. The cluster analyses shown in Figure 1 identified seven main clusters. Unique clusters for *Methylocella/Methylocapsa*, *M. sporium* and *M. trichosporium*, plus four separate *Methylocystis* clusters could be distinguished. MDS (Figure 2) confirms this clustering. In Figure 3, overlays are presented with the values (% of total PLFA) of five PLFA exhibiting the most variation among the type II clusters identified in Figure 1. *M. sporium* profiles are more similar to *Methylocella/Methylocapsa* than to *M. trichosporium* because they lack the PLFA C18:1ω8c. All *M. sporium* strains tested contained C18:1ω7c as the only C18 PLFA. The relatively high amounts of C16:1ω7c were also a factor separating *Methylosinus*

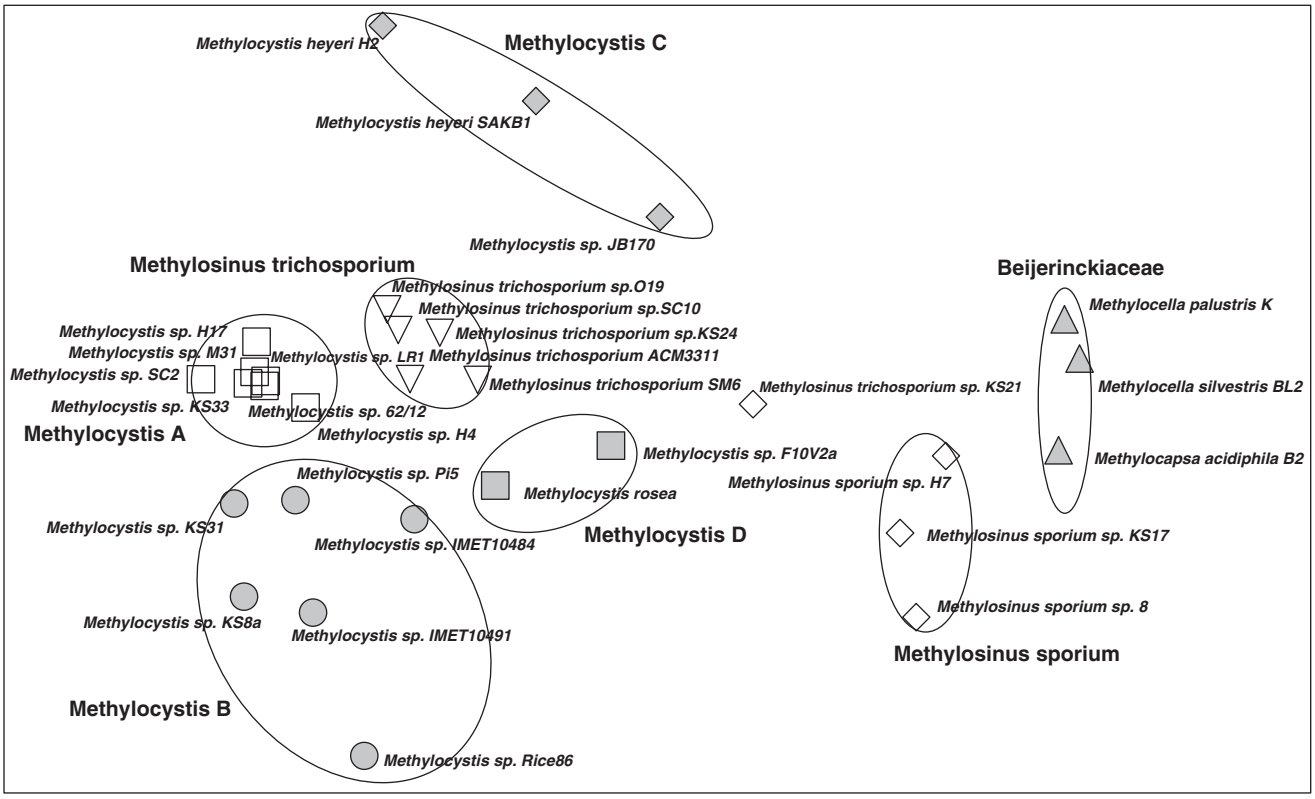

Figure 2 Multivariate statistical analyses of phospholipid ester-linked fatty acids (PLFA) profiles of type II methanotrophs grown on methane. The figure combines data obtained in this study with data from the literature (see also Supplementary Table 1a+b). The input of the MDS (multidimensional scaling) analyses was a Bray–Curtis similarity matrix of square-root-transformed PLFA profiles (% of total PLFA content). The two-dimensional arrangement of datapoints represents relative differences in similarity and does not display the absolute differences.

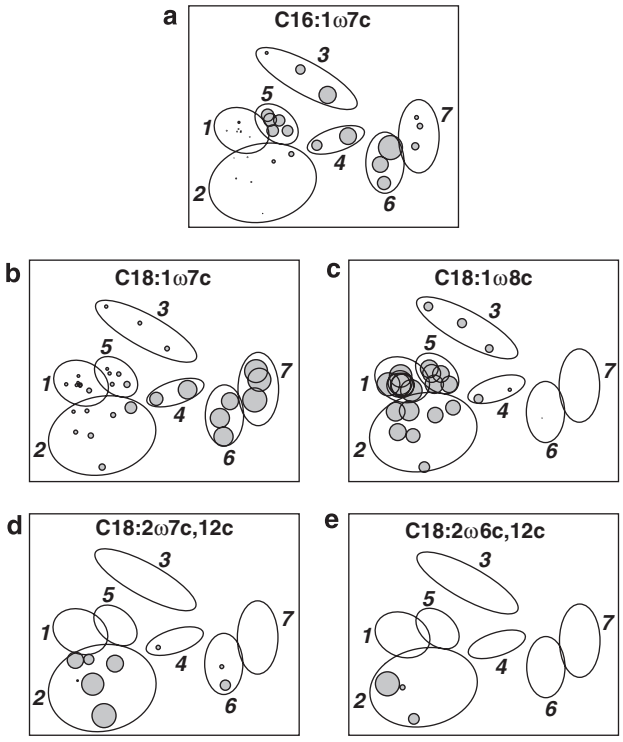

Figure 3 Multivariate statistical analyses of phospholipid ester-linked fatty acids (PLFA) profiles of type II methanotrophs grown on methane. The figure combines data obtained in this study with data from the literature (see also Supplementary Table 1a+b). The input of the MDS (multidimensional scaling) analyses was Bray–Curtis similarity matrices of square-root-transformed PLFA profiles (% of total PLFA content). The symbols depict the values of individual PLFA (C16:1ω7c (**a**), C18:1ω7c (**b**), C18:1ω8c (**c**), C18:2ω7c,12c (**d**) and C18:2ω6c,12c (**e**)) exhibiting the most variation between the type II clusters (1 = *Methylocystis* A; 2 = *Methylocystis* B; 3 = *Methylocystis* C; *Methylocystis* D; 5 = *Methylosinus trichosporium*; 6 = *Methylosinus sporium*; 7 = *Beijrinckiaceae*).

species from *Methylocystis* groups A and B. Four *Methylocystis* groups could be distinguished based on the presence of large amounts of C16:1ω8c (group C), equal amounts of C18:1ω8c and C18:1ω7c in combination with C16:1ω7c (group D), almost exclusively C18:1ω8c (group A), or the presence of the novel C18:2 PLFAs (group B).

PLFA database of all known types I and II MB

Figure 4 presents a cluster analyses of all available PLFA profiles of methanotrophs. Cluster analyses and MDS both show a primary separation of the three major taxonomic groups: *Verrucomicrobia, Gammaproteobacteria* (type I) and *Alphaproteobacteria* (type II). *Methylohalobius* is the only outlier, this type I MB clusters with the type II MB PLFA profiles in the Bray–Curtis analysis (Figure 4), although the MDS properly groups it with other type I MB (data not shown). In total 18 groups can be distinguished. Within the type I MB the mesophilic genera are clearly separated from the thermophilic genera *Methylothermus*, *Methylococcus and Methylocaldum*. The only *Verrucomicrobia* representative for which a PLFA profile is available groups distantly from both proteobacterial clusters, although it is somewhat closer to the type I MB.

Within the type II MB the two genera of the family *Beijerinckaceae* (*Methylocella* and *Methylocapsa)* group into a single cluster, and together with *M. sporium* strains are separated from the various *Methylocystis* groups and the *M. trichosporium* group. Again there is a single exception: *M. trichosporium* strain KS21 clusters with *M. sporium* rather than with other *M. trichosporium* strains. However, it was already noted that the sequence-based phylogenetic assignment of KS21 to the species *trichosporium* was very tenuous, and may be incorrect (Heyer *et al*., 2002). The *Methylocystis heyeri* strains also have a distinct position within the type II profiles due to the presence of a PLFA once considered to be type I specific (C16:1ω8c).

PLFA content of type II MB

Methylocystis strains contained on average 213.26 ± 23.27 (s.e.) μmol of PLFA per gram of dry cell material. This was significantly higher (*t*-test, $P<0.05$) than *M. sporium* strains, which had on average 84.00 ± 44.87 (s.e.) μmol PLFA per gram of dry cell material. The PLFA content of *M. trichosporium* strains was intermediate (132.56 ± 33.95 μmol PLFA per gram of dry cell material) and did not differ significantly from *Methyloystis* and *M. sporium* strains.

Effect of substrate type on PLFA profiles

Three *M. trichosporium* strains were grown on methane, methanol or methane + methanol. No PLFA appeared or disappeared as the consequence of changing the growth substrate. Slight differences in the relative amounts of various PLFA between the treatments were observed (Figure 5). Strains O19 and SC10 had lower amounts of C16:0 and higher amounts of C18:1ω8c when grown with methane alone. Strain KS24 had higher amounts of C18:1ω7c when methanol was present as a substrate.

Seven other type II species were grown on both methane and methanol as shown in Table 1. Again the growth substrate had hardly any effect on the PLFA profiles. Application of the ANOSIM test of similarity matrices of the profiles obtained with methane and methanol revealed no statistically significant difference between profiles (global R 0.044, $P<0.173$).

Discussion

Implications of novel PLFA features of Alphaproteobacteria

The analysis of PLFA profiles generated from several newly isolated type II methanotrophs allows a greatly improved level of chemotaxonomic resolution

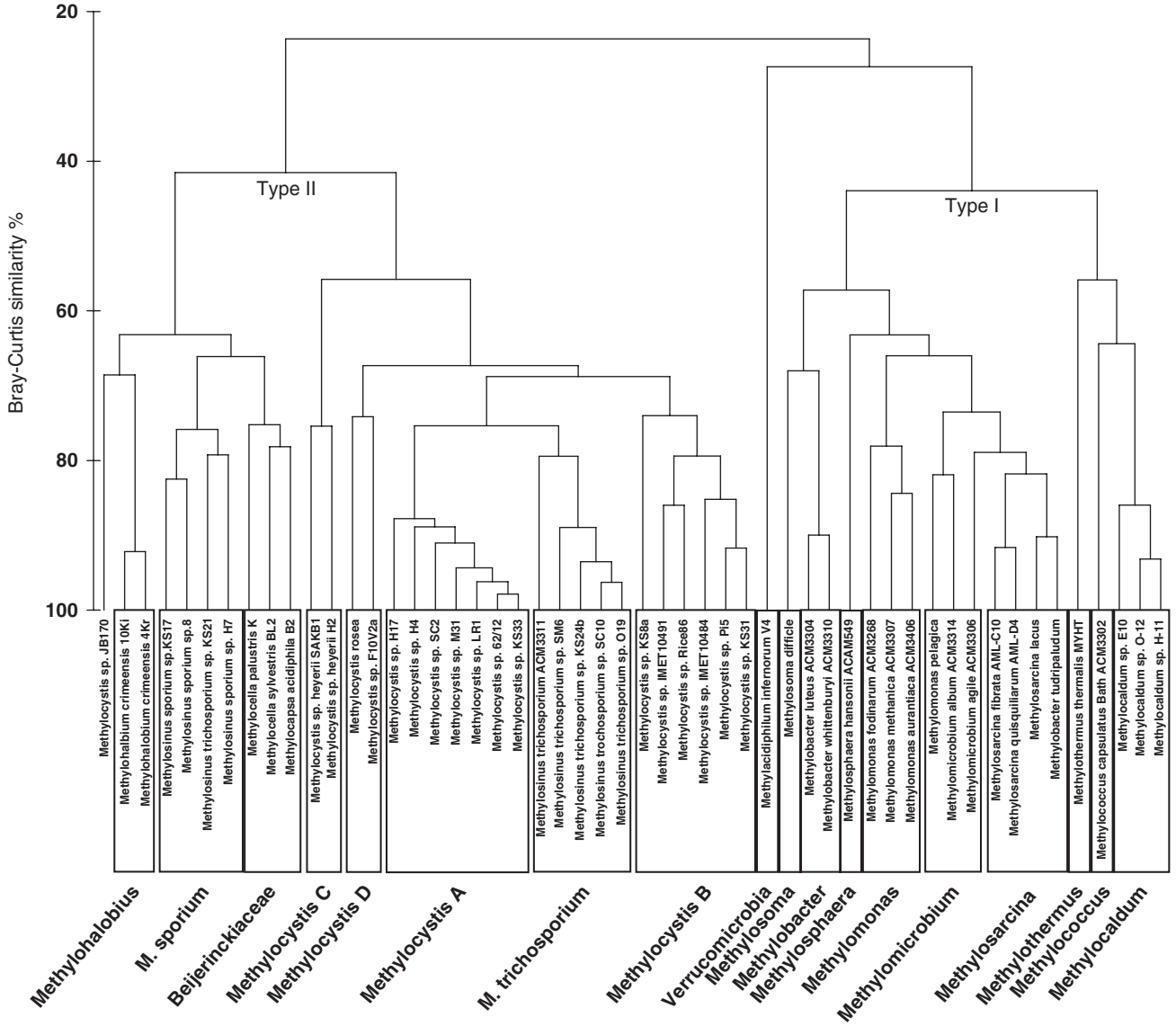

Figure 4 Multivariate statistical analyses of phospholipid ester-linked fatty acids (PLFA) profiles of methanotrophs belonging to the taxa *Gammaproteobacteria* (type I), *Alphaproteobacteria* (type II) or *Verrucomicrobia*. The figure combines data obtained in this study with data from the literature (see also Supplementary Table 1a). The input of the cluster analyses were Bray–Curtis similarity matrices using PLFA profiles (% of total PLFA content). The clustering was made using the Group Average linking Cluster analyses (UPGMA).

of MB. Using these new data in combination with the PLFA profiles of types I and II MB already available, we constructed a comprehensive database, which can serve as a framework for linking activity to identity in SIP-PLFA analyses of environmental samples.

MB have been studied intensively in many habitats (Hanson and Hanson, 1996; Conrad, 2007; Dunfield, 2007), not only because of their environmental relevance but also because most are obligate in nature—that is, they live only methylotrophically. This makes them excellent model organisms for microbial ecology studies, because a direct link can be made between a biogeochemical process and a taxonomically defined group of bacteria. The use of CH_4 as a carbon and energy source by these bacteria enables ecologists to apply isotopically labelled CH_4 as a tracer that is incorporated into taxonomically relevant compounds like PLFA or DNA/RNA (compare McDonald *et al.*, 2008). Many studies have combined the use of the stable isotope ^{13}C or the radioisotope ^{14}C with analyses of PLFA to identify what MB are active in an environment (for example, Boschker *et al.*, 1998; Roslev *et al.*, 1998; Bodelier *et al.*, 2000; Knief *et al.*, 2003, 2006; Evershed *et al.*, 2006; Mohanty *et al.*, 2006; Deines *et al.*, 2007; Shrestha *et al.*, 2008; Crossman *et al.*, 2005). However, in these studies, the effects of environmental factors on MB communities have generally only been assessed at the broad level of type I versus type II species, by comparing label incorporation into C16 (type I) or C18 (type II) PLFA.

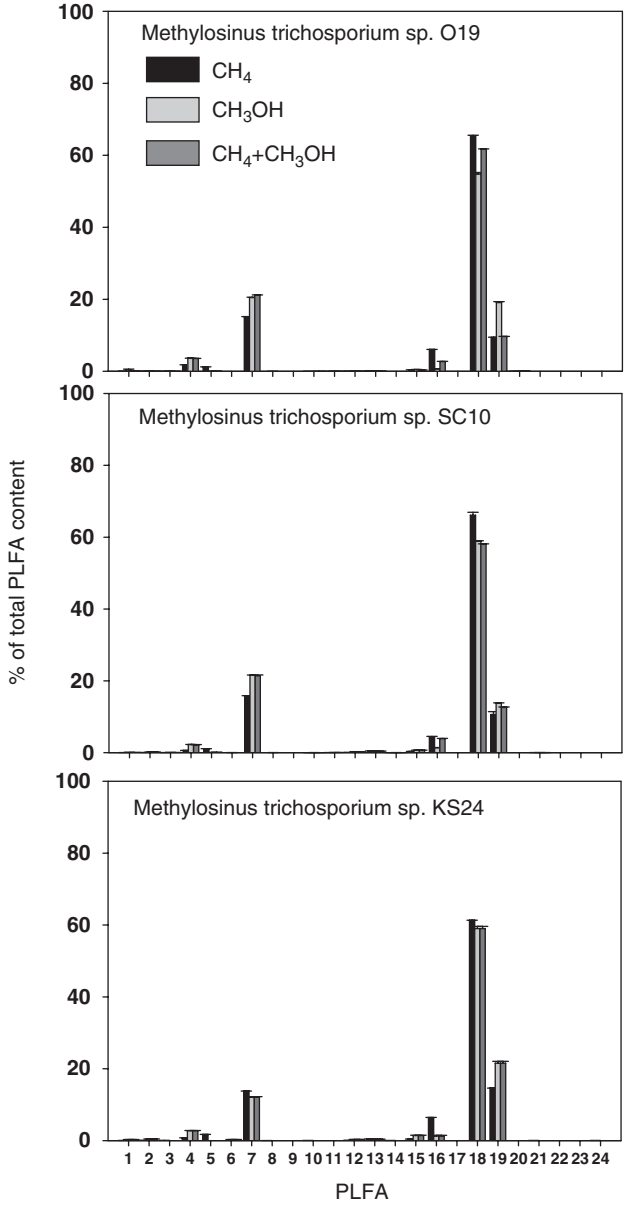

Figure 5 Phospholipid ester-linked fatty acids (PLFA) profiles (% of total PLFA content) of three *Methylosinus trichosporium* strains grown on methane, methanol or methane + methanol. Bars indicate the means (+1sd) of 2 replicate GC-MS analyses. Only PLFA contributing more than 0.1% of the total are depicted. PLFA are numbered: (1) C14:0, (2) iC15:0, (3) C15:0, (4) C16:0, (5) C16:1ω9t, (6) C16:1 ω5t, (7) C16:1ω7c, (8) C16:1ω6c, (9) C16:1ω5c, (10) aC17:0, (11) iC17:1ω7c, (12) C17:0, (13) cyC17:0, (14) C17:1ω6c, (15) C18:0, (16) C18:1ω9t, (17) C18:1ω9c, (18) C18:1ω8c, (19) C18:1ω7c, (20) C18:1ω6c, (21) C18:1ω5c, (22) C18:2ω7c,12c, (23) C18:2ω6c,12c, (24) cyC19:0.

Only two studies have used multivariate analyses to compare environmental PLFA profiles to known MB isolates (Maxfield *et al.*, 2006; Crossman *et al.*, 2005). However, the cluster analyses made by these authors were referenced against the PLFA profiles of MB cultures available to that date, which had a very poor resolution at the subgenus level and treated all *Methylocystaceae* (*M. sporium/trichosporium* and *Methylocystis* spp.) as being identical. Most available data on type II MB PLFA profiles were determined three decades ago (Nichols *et al.*, 1985; Bowman *et al.*, 1991; Bowman *et al.*, 1993). Isolation and description of a large number of new strains (Heyer *et al.*, 2002) offered the opportunity to expand the database. The analyses of a representative number of stains of this collection resulted in the detection of a number of features subdividing the type II species. The most prominent are two novel C18:2 PLFA which occur in a number of *Methylocystis* strains. These PLFA have previously been found only in representatives of the phylum *Planctomycetes* (Kulichevskaya *et al.*, 2008) isolated from acidic peat. Hence, these PLFA might be hypothesized to represent a recent adaptation to the environmental conditions of peat (Guerzoni *et al.*, 2001). However considering that these strains, designated as *Methylocystis* group B, also separate from other *Methylocystis* strains on the basis of 16S rRNA and *pmoA* gene phylogenies (Heyer *et al.*, 2002), and that some were obtained from soil and lake sediments, makes this hypothesis unlikely.

Another marked feature is the absence of C18:1ω8c in all *M. sporium* strains investigated. Consequently, the profiles of *M. sporium* strains are very similar to those of *Methylocella* and *Methylocapsa*. The genus *Methylosinus* is still described as having C18:1ω8c as the dominant PLFA (Bowman *et al.*, 1993; Bowman, 2006). Hence, identifying the active MB species in SIP-PLFA labelling studies where the profile is dominated by C16:0 + C16:1ω7c + C18:1ω7c is problematic as it may indicate either *Methylocella/Methylocapsa* or *M. sporium*. For example, atmospheric methane incorporation in forest soil has been assigned to *Methylocapsa*-related MB on the basis of PLFA-SIP, because most of the label was detected in C18:1ω7c (Maxfield *et al.*, 2006; Singh *et al.*, 2007). However, the data are equally indicative of *M. sporium*-like bacteria. *Methylosinus sp. KS21* also contains a C17-PLFA that is sometimes labelled in SIP studies investigating atmospheric consumption of methane (Bull *et al.*, 2000). Although *M. sporium* are very rarely detected in forest soils, and *pmoA* recovery efforts strongly support the primary importance of *Methylocapsa*-like strains (Dunfield, 2007), a role for *M. sporium* in atmospheric methane consumption cannot be ruled out on the basis of PLFA analyses. Additional assessment could be done by RNA-SIP or DNA-SIP followed by 16S rRNA or *pmoA* gene fingerprinting analyses.

Extended PLFA database and molecular phylogeny

Taking previously analysed PLFA profiles together with those analysed in this study, it is obvious that PLFA profiles give a good representation of the taxonomy of MB as based on 16S rRNA and *pmoA* gene sequence phylogenetic analyses (Heyer *et al.*, 2002). All genera are well separated from each other and properly assigned to type I or type II, with the

Table 1 PLFA profiles (% of total PLFA) of seven methanotrophic strains grown on methane or methanol

	Species													
	Methylocystis *sp. IMET10484*		Methylocystis *sp. Pi5*		Methylocystis *sp. 62/12*		Methylocystis *sp. SC2*		Methylocystis *sp. F10V2a*		Methylosinus sporium *sp. KS17*		Methylosimnus trichosporium *sp. SM6*	
	Substrate													
PLFA	CH_4	CH_3OH	CH_4	CH_3OH	CH_4	CH_3OH	CH_4	CH_3OH	CH_4	CH_3OH	CH_4	CH_3OH	CH_4	CH_3OH
C14:0	0.12 (0.00)	0.29 (0.25)		0.18 (0.00)		0.51 (0.00)			0.12 (0.00)	0.29 (0.25)	0.11 (0.00)	0.24 (0.02)	0.14 (0.00)	0.17 (0.00)
iC15:0	0.27 (0.00)	0.29 (0.04)				0.26 (0.02)			0.27 (0.00)	0.29 (0.03)	0.40 (0.04)	0.45 (0.01)	0.23 (0.01)	0.12 (0.01)
C15:0	0.11 (0.00)	0.11 (0.01)				0.27 (0.00)		0.25 (0.07)	0.10 (0.00)	0.11 (0.01)				
C16:0	2.66 (0.00)	4.57 (2.68)	0.48 (0.00)	1.12 (0.00)	0.49 (0.00)	3.10 (0.00)	0.30 (0.00)	1.08 (0.18)	2.66 (0.00)	4.57 (2.68)	1.23 (0.03)	2.21 (0.01)	1.02 (0.02)	4.95 (0.03)
C16:1ω9t						0.12 (0.00)						0.42 (0.00)	0.36 (0.00)	0.13 (0.00)
C16:1ω5t				0.18 (0.01)		0.61 (0.06)		0.55 (0.42)				0.14 (0.00)		
C16:1ω7c	12.43 (0.00)	14.36 (2.71)	0.63 (0.02)	0.67 (0.02)	0.73 (0.00)	0.68 (0.04)	0.66 (0.02)	1.13 (0.64)	12.43 (0.00)	14.35 (2.71)	16.10 (0.68)	24.37 (0.01)	12.97 (0.09)	20.53 (0.06)
C16:1ω6c	0.15 (0.00)	0.12 (0.04)			0.21 (0.00)	0.32 (0.03)	0.12 (0.00)	0.15 (0.22)	0.14 (0.00)	0.12 (0.04)	1.33 (0.02)	0.18 (0.00)		
C16:1ω5c														
aC17:0						0.14 (0.00)		0.52 (0.25)			0.11 (0.00)			
iC17:1ω7c								0.47 (0.67)			0.43 (0.02)	0.51 (0.01)		
C17:0				0.13 (0.00)		0.31 (0.00)		0.12 (0.16)			0.27 (0.02)	0.19 (0.00)	0.16 (0.00)	0.17 (0.00)
cyC17:0	0.22 (0.00)	0.21 (0.03)				0.13 (0.00)			0.23 (0.00)	0.21 (0.03)	1.28 (0.01)	0.41 (0.05)	0.65 (0.00)	0.21 (0.00)
C17:1ω6c											0.27 (0.01)			
C18:0	0.15 (0.00)	0.16 (0.01)	0.48 (0.01)	0.76 (0.00)	0.25 (0.00)	1.57 (0.00)		3.68 (3.83)	0.15 (0.00)	0.16 (0.01)	1.37 (0.05)	1.30 (0.06)	0.69 (0.01)	0.50 (0.00)
C18:1ω9t	0.71 (0.08)	0.72 (0.08)	1.11 (0.01)	1.22 (0.01)	1.45 (0.00)	1.98 (0.04)	0.91 (0.12)	2.83 (1.08)	0.71 (0.07)	0.72 (0.08)	0.26 (0.24)	1.42 (0.00)	2.36 (0.04)	1.94 (0.01)
C18:1ω9c							1.88 (0.09)	1.45 (0.25)			0.18 (0.04)	0.24 (0.00)		
C18:1ω8c	33.06 (0.10)	43.25 (14.51)	72.25 (0.52)	69.50 (0.28)	85.73 (0.42)	81.31 (0.11)	84.65 (0.29)	66.08 (5.44)	33.06 (0.10)	43.25 (14.51)			60.33 (0.49)	55.34 (0.28)
C18:1ω7c	45.10 (0.19)	31.67 (19.17)	13.10 (0.16)	16.67 (0.39)	10.79 (0.42)	8.04 (0.07)	10.87 (0.32)	16.91 (0.18)	45.10 (0.19)	31.67 (19.17)	65.67 (0.30)	58.49 (0.11)	20.88 (0.32)	15.71 (0.26)
C18:1ω6c												0.23 (0.05)		
C18:1ω5c			0.24 (0.00)	0.23 (0.00)				0.18 (0.26)						
C18:2ω7c,12c	4.46 (0.00)	3.69 (1.08)	10.44 (0.16)	8.78 (0.01)		0.50 (0.00)			4.46 (0.00)	3.69 (1.08)	10.54 (0.25)	8.738 (0.00)		
C18:2ω6c,12c												0.17 (0.01)		
cyC19:0														

Abbreviation: PLFA, phospholipid ester-linked fatty acids.
Only values >0.1% of total PLFA are indicated. Values are the mean of two replicate GC-MS analyses per extracted strain. In brackets the standard deviations of these replicate analyses are indicated.

possible exception of *Methylohalobius*. The hypersaline habitat of this halophile may have exerted selection pressure for an unusual PLFA profile (Heyer *et al.*, 2005). The PLFA data also provides supporting taxonomic evidence for some species that could not unequivocally be assigned to a species on the basis of 16S rRNA and *pmoA* genes (Heyer *et al.*, 2002). *Methylosinus* sp. KS21 could not reliably be assigned to a species based on sequence phylogeny, but PLFA analysis identifies it as being more similar to *M. sporium* than to *M. trichosporium*. Unclear affiliation of *Methylocystis* sp. F10V2a by gene phylogenies (Heyer *et al.*, 2002) was also mirrored by an unusual PLFA profile.

In combination with stable isotope labelling, PLFA profiles are a useful tool to classify the active MB species in environmental samples down to the genus and in some cases to the species level. Using the improved database, this method should be particularly useful in environments where only a few species are the dominant methane metabolizers (Deines *et al.*, 2007). When types I and II MB are both active at the same time, classification using labelling becomes problematic due to overlapping PLFA. This can be overcome by performing cluster analyses of environmental samples where either PLFA exclusive for type I or type II (that is, PLFA that account for less than 0.1% of the total PLFA in one or the other type; Mohanty *et al.*, 2006) are taken into consideration. Complementing this approach by SIP labelling of DNA/RNA in parallel will increase phylogenetic resolution, correct possible overlap in active cultures and account for activity of yet-uncultivated MB (Qiu *et al.*, 2008).

Effect of substrate and MB-specific PLFA as measures of abundance

The profiles are also stable with respect to substrate usage. Incubating strains with methane, methanol or a combination of both did not lead to significant changes in the profiles. This experiment was done in part to see whether variable profiles observed in environmental samples like forest soils could be explained by bacteria growing on methanol *in situ* instead of methane. However, this was not the case. Of course PLFA profiles of individual bacterial species can change with varying environmental parameters (compare (Alvarez-Ordonez *et al.*, 2008). For methanotrophs this has not yet been studied but it seems unlikely considering the tight relationship between PLFA profiles of MB and their DNA-based phylogeny.

As the specific PLFA C16:1ω8c and C18:1ω8c have only been found in MB to date, the concentrations of these PLFA in environmental samples are sometimes used to determine population sizes of types I and II MB (for example, Sundh *et al.*, 1995; Macalady *et al.*, 2002; Mohanty *et al.*, 2006). These calculations are based on a number of assumptions, such as the PLFA content of individual MB cells. Sundh *et al.* (1995) assumed a total PLFA content of methanotrophs of 100 μmol per gram dry weight. However, our data show that *Methylocystis* strains can have twice that amount of PLFA. Hence, the above assumption can lead to underestimation of type II MB population sizes in communities dominated by *Methylocystis*. Moreover population assessment based on specific PLFA abundance in communities dominated by *M. sporium*, *Methylocella* spp., *Methylocapsa* spp. or the recently discovered *Verrucomicrobia* methanotrophs will be largely incorrect because of the lack of signature PLFAs in these species.

Conclusion

This study enhances the applicability of PLFA profiles as a tool to taxonomically characterise active MB species *in situ*. The resolution of type II MB is significantly enhanced, which should lead to taxonomic assignment with higher confidence in methane-rich as well as methane-poor habitats. In combination with molecular community analyses, tools like SIP-PLFA offer possibilities to link function and identity in communities of MB that are unique in the field of environmental microbiology.

Acknowledgements

We thank Peter Nichols, John Bowman and Mette Svenning for providing data of non-publicly available details of MOB PLFA profiles. Sergey Stoliar and Mary Lidstrom are kindly thanked for providing reference cultures. This study was part of the ESF-Eurodiversity programme (ERAS-CT-2003-98049, 6th EU-framework programme) and was financially supported by grants from the Netherlands Organization for Scientific Research (NWO; Grant no. 855.01.108), and the Max-Planck Society.

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Supplementary Information accompanies the paper on The ISME Journal website (http://www.nature.com/ismej)