Prerequisites for amplicon pyrosequencing of microbial methanol utilizers in the environment

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The commercial availability of next generation sequencing (NGS) technologies facilitated the assessment of functional groups of microorganisms in the environment with high coverage, resolution, and reproducibility. Soil methylotrophs were among the first microorganisms in the environment that were assessed with molecular tools, and nowadays, as well with NGS technologies. Studies in the past years re-attracted notice to the pivotal role of methylotrophs in global conversions of methanol, which mainly originates from plants, and is involved in oxidative reactions and ozone formation in, and atmosphere. Aerobic methanol utilizers belong to Bacteria, yeasts, Ascomycota, and molds. Numerous bacterial methylotrophs are facultatively aerobic, and also contribute to anaerobic methanol oxidation in the environment, whereas strict anaerobic methanol utilizers belong to methanogens and acetogens. The diversity of enzymes catalyzing the initial oxidation of methanol is considerable, and comprises at least five different enzyme types in aerobes, and one in strict anaerobes. Only the gene of the large subunit of pyroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (MDH; mxaF) has been analyzed by environmental pyrosequencing. To enable a comprehensive assessment of methanol utilizers in the environment, new primers targeting genes of the PQQ MDH in Methylbium (mxaF), of the nicotinamide adenine dinucleotide-dependent MDH (mfdh), of the methanol oxidoreductase of Actinobacteria (mdh), of the fungal flavin adenine dinucleotide-dependent alcohol oxidase (moxF), mox2, and homologs, and of the gene of the large subunit of the methanol:corrinoid methyltransferases (mtaC) in methanogens and acetogens need to be developed. Combined stable isotope probing of nucleic acids or proteins with amplicon-based NGS are straightforward approaches to reveal insights into functions of certain methylotrophic taxa in the global methanol cycle.

Keywords: methylotroph, PQQ MDH, PQQ MDH2, NAD MDH, FAD AO, mxaF, moxF, mtaC

METHYLOTROPHS IMPACT ON GLOBAL ONE-CARBON COMPOUND CYCLING

Aerobic methylotrophs occur in terrestrial and aquatic environments on the whole planet, and have been detected in aerated and flooded soils of wetlands, grasslands, tundra, and deserts, and occur in the phyllosphere and rhizosphere of plants, in open ocean waters and other marine habitats (Giovannoni et al., 2008; Angel and Conrad, 2009; Kolb, 2009a; Witzke et al., 2011; Bassett et al., 2012; Gupta et al., 2012; He et al., 2012; Knief et al., 2012a; Vehlert, 2012) suggesting that their unique physiology that allows them to utilize reduced C1 compounds as carbon and energy source is of global relevance in ecosystems. Methylotrophs ubiquitously occur in terrestrial ecosystems, i.e., likely, since plants produce C1 compounds. Growing plants emit methanol (up to 0.2% of the photosynthetic carbon) and traces of chloromethane and methane (Keppler et al., 2005; Keppler et al., 2006), during decay of lignocellulosic plant material methanol is released (Gallaway and Kristine, 2002), and plant compounds are eventually converted to methane under anoxic conditions (Drake et al., 2012; Gupta et al., 2012; Knief et al., 2012a; Vehlert, 2012) suggesting that their unique physiology that allows them to utilize reduced C1 compounds as carbon and energy source is of global relevance in ecosystems. Methylotrophs ubiquitously occur in terrestrial ecosystems, i.e., likely, since plants produce C1 compounds. Growing plants emit methanol (up to 0.2% of the photosynthetic carbon) and traces of chloromethane and methane (Keppler et al., 2005; Keppler et al., 2006), during decay of lignocellulosic plant material methanol is released (Gallaway and Kristine, 2002), and plant compounds are eventually converted to methane under anoxic conditions (Drake et al., 2012; Gupta et al., 2012; Knief et al., 2012a; Vehlert, 2012).
C1 compounds are highly volatile and thus, are emitted into the atmosphere. Consequently, the two most abundant organic compounds in the atmosphere are methane and methanol (Foerster et al., 2007). The steady-state concentration of methanol in the atmosphere (1–10 ppb) is about 1,000-fold lower than that of atmospheric methane (1,800 ppb; Gallab and Kirstine, 2002; Heikes et al., 2002; Jacob et al., 2003). Whereas, the estimated global emission rate of methane (~15 Tmol per year) from terrestrial ecosystems is only twice as high as the global terrestrial emission rate of methanol (~5 Tmol per year) indicating that methanol is substantially more susceptible to atmospheric chemical reactions (Gallab and Kirstine, 2002; Jacob et al., 2005; Kolb, 2009a). Methanol triggers the formation of tropospheric ozone, and has indirectly a threefold higher global warming potential on a one-hundred-year basis than carbon dioxide (Foerster et al., 2007).

Release of methane from terrestrial ecosystems into the atmosphere is reduced by aerobic methylotrophs (Conrad, 1995). Many aerated soils in natural ecosystems are even net sinks for atmospheric methane, which is often correlated with the predominance of certain genotypes, such as USCα (Dunfield, 2007; Kolb, 2009b). Methanotrophic methylotrophs have been addressed in numerous environmental studies by using gene markers and other biomarkers, and are one of the most studied functional groups of microorganisms in the environment (e.g., Dedysh, 2009; Kolb, 2009b; Dörr et al., 2010; Lüke et al., 2010; Luke and Fresnel, 2011). There are more than 400 publications on methanotrophs in ecosystems over the past 25 years based on keyword searches in literature databases (Web of Knowledge, 04.07.2013; http://apps.webofknowledge.com) highlighting the interest in understanding the role of methanotrophs in the global carbon cycle.

Non-methanotrophic methylotrophs likely have a similar importance for the global methane cycle, a fact that has recently been more thoroughly addressed in the phyllosphere, soil, and ocean waters (Délomotte et al., 2009; Kolb, 2009b; Knief et al., 2010; Smirnov et al., 2011; Vorholt, 2012; Stacheter et al., 2013). The assessment of methanol-utilizing methylotrophs in the environment is less straightforward than the detection of methanotrophs, since methanol utilizers have a substantially larger diversity than methanotrophs, and the enzymes that catalyze the diagnostic reaction, i.e., the oxidation of methanol to formaldehyde, are more diverse than methane monooxygenases making the detection of non-methanotrophic methanol utilizers more challenging (Chistoserdova et al., 2009; Kolb, 2009a; Stacheter et al., 2013).

The role of methylotrophs in global methanol cycling is still scarcely investigated and warrants studies that address the response, activity, and distribution of methanol utilizers in terrestrial and other environments. Hence, suitable gene targets are mandatory to analyze methanol-utilizing microorganisms with amplicon pyrosequencing or to detect them in metagenomic, transcriptomic, and proteomic datasets based on sequence homology. The review will describe the latest knowledge on microbial taxa that are capable of methanol oxidation including those organisms that putatively utilize methanol under anoxic conditions, and will identify gene markers that have been and can be employed for analysis of PCR amplicons by high-throughput NGS techniques.

**FACULTATIVELY AEROBIC METHANOL UTILIZERS**

Microorganisms that have the capability to utilize methanol with molecular oxygen as an electron acceptor belong to various phyla of Bacteria, and have been found within yeast, mold fungi, and Ascomycota (Table 1; Bystrýk et al., 1988; Trottenko and Bystrýk, 1999; Nakagawa et al., 1996; Nozaki et al., 1996; Silva et al., 2009; Sipic et al., 2012). Bacterial methanol utilizers belong to Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, Flavobacteria, Bacilli, and Actinobacteria. Yet, methanol utilization (MUT) among Archaea only occurs in strict anaerobic methanogens. Generally, it is known that several bacterial methylotrophs utilize methanol or other C1 compounds for dissimulation, but cannot assimilate carbon from C1 compounds. Strain HTCC2181 is a recent example, which demonstrates this strategy of C1 compound utilization (Giovannoni et al., 2008; Halsey et al., 2012). Over 200 aerobic species of methylotrophic Bacteria have been described (Tables 1 and 2; Kolb, 2009a). Most of the known isolates are Gram-negative. Thus, it is remarkable that a second isolate of the genus Bacillus has recently been described, which was not enriched on conventional methylotroph media suggesting a largely uncovered diversity of Gram-positive methanol utilizers in the environment (Table 2; Ling et al., 2011).

It is well established that some facultatively aerobic methanol utilizers are capable of growth on C1 compounds with nitrate as an electron acceptor (Kolb, 2009a). In addition, many more methylotrophs that have the ability to use nitrate as an alternative electron acceptor have not yet been tested for anaerobic methanol oxidation (Bansforth and Quayle, 1978; Kolb, 2009a); recent examples, in which the physiology has been thoroughly assessed, are Methylosinus universus FAMS, and Methylosinus versutus (Kalyuzhnaya et al., 2012; Lu et al., 2012; Mustafeev et al., 2013). In environments with a high nitrogen input (for example by fertilization) and turnover, facultative aerobic and nitrate-dependent degradation of methanol likely occurs in oxygen-limited zones (Lu et al., 2012). Based on the current knowledge, these organisms are accessible by the same gene markers as described in the following section (Figure 1).

**MARKER GENES OF BACTERIAL METHANOL UTILIZERS**

Amplification-based analysis of the diversity of methanol utilizers can be achieved by deep sequencing of genes that are diagnostic for methanol oxidation (Figure 1; Stacheter et al., 2013). The C1 metabolism of bacterial methanol utilizers comprises a series of enzymatic reactions, which partially cannot be found in other heterotrophs and are thus, diagnostic for methylotrophs. The most characteristic enzymatic step is the initial oxidation of methanol to formaldehyde (Figure 1). The oxidation of methanol can be catalyzed by at least three different enzymes in Bacteria. There is a pyrroloquinoline quinone (PQQ)-dependent and a nicotinamide adenine dinucleotide (NAD)-dependent methanol dehydrogenase (MDH, Devries et al., 1992; Chistoserdova et al., 2009; Kung et al., 2013). PQQ MDH occurs in Gram-negative Bacteria, whereas
Table 1 | Classes and phyla of Bacteria and fungi that contain methanol-utilizing methylotrophs based on previous reviews (Kolb, 2009b; Grewe et al., 2012).

| Class/phylum/order | Representative species |
|--------------------|------------------------|
| Actinobacteria      |                        |
| Brevibacteriaceae   | Brevibacterium casei    |
| Micrococcaceae      | Arthrobacter methylotrophus |
| Mycobacteriaceae    | Mycobacterium gastrin   |
| Nocardiaceae        | Rhodococcus erythropolis |
| Pseudonocardiales   | Amycolatopsis methanolica |
| Bacilli             | Bacillus methanolicus   |
| Alphaproteobacteria |                        |
| Acinetobacteriaceae | Acinetobacter baumannii |
| Beijerinckiaceae    | Methylcoccus aurina     |
| Bradyrhizobiaceae   | Alcaligenes faecalis    |
| Hyphomicrobiaceae   | Anguillomicrobium teutonicus |
| Methylocystaceae    | Methylocystis extorquens |
| Methylophilaceae    | Methylophilus glucosoxidans |
| Methylophagaceae    | Mesorhizobium lotii    |
| Rhizobiaceae        | Ensifer methanolicus    |
| Rhodobacteraceae    | Paracoccus alkenifer    |
| Sphingomonadaceae   | Sphingomonas melonis    |
| Xanthobacteraceae   | Xanthobacter ambivalens |
| Betaproteobacteria  |                        |
| Comamonadaceae      | Vibrio parahaemolyticus |
| Methyloidaceae      | Methylosinus glutinolique |
| Rhodocyclaceae      | Rhodococcus rhodochrous |
| Burkholderiaceae    | Methylococcus capsulatus |
| Gammaproteobacteria |                        |
| Enterobacteriaceae  | Klebsiella oxytoca      |
| Methylococaceae     | Methyloccus capsulatus  |
| Pseudomonadaceae    | Pseudoalteromonas aeruginosa |
| Vibrioaceae         | Photobacterium indigens |
| Classification unclear | Methylobacterium lacus |
| Ascomycota          |                        |
| Glotrichellaceae    | Gilvocladium deliquescens |
| Paecilomyces variotii | Paecilomyces variotii |
| Trichoderma lignorum |
| Yeasts              |                        |
| Candida (any)       | Candida albicans (any)  |
| Hansenula capsulata (any) | Hansenula capsulata (any) |
| Pichia pastoris (any) | Pichia pastoris (any) |
| Mold fungi          |                        |
| Paecilomyces variotii | Paecilomyces variotii |
| Penicillium chrysogenum |

A-factors mxaF-like gene mxaF-like. Growth on methanol has not been tested.

The NAD MDH is typical of Gram-positive Bacillus strains and is encoded by the gene mxaF (Chistoserdova et al., 2009). Furthermore, in Gram-positive Actinobacteria (Amycolatopsis methanolica, Mycobacterium gastri MB19) a methanol:NDMA (N,N'-dimethylyl-nitrosamine) oxidoreductase (MDO) has been reported (Bystrykh et al., 1993; Vanoppen et al., 1993; Park et al., 2010).

The gene mxaF encodes the catalytic subunit of PQQ MDH, which is composed of two different subunits (i.e., MxaF1). However, there is a distinctly related homolog in some methylotrophic Burkholderiales (Methylibium, i.e., the gene was named mxaF2), which encodes an alternative PQQ-dependent MDH (Kalmykina et al., 2008). Beyond mxaF2, a further homolog of mxaF is known, i.e., xoxF, for which a functional role in methanol-metabolism is under debate. xoxF-like genes (synonymous to mxaF) occur in Bradyrhizobiaceae and other rhizobia, and may encode functional MDHs (Vitiyanto et al., 2011). Similar genes can be frequently detected in soil microbial communities using mxaF-specific primers (Table 1, Stacheter et al., 2013). If rhizobia that do not possess the classical PQQ MDH (i.e., MxaF; Mosovi et al., 2005), also utilize and grow on methanol has not systematically been analyzed yet. Nonetheless, Bradyrhizobium sp. MAFF211645 contains a Ce+ inducible XoxF-like MDH (Vitiyanto et al., 2011). The first functional proof of XoxF as a MDH was demonstrated in the phototroph Rhodobacter sphaeroides (Wilson et al., 2008). Studies on Methylobacterium extorquens AM1 suggest that XoxF1 and XoxF2 are involved in the regulation of mxaF (Schmidt et al., 2010; Skovran et al., 2011). Purified XoxF1 of Methylobacterium extorquens AM1 has highest methanol oxidation activities when cells were grown with methanol and 30 μM La+3. These activities were comparable to the canonical PQQ MDH MxaF1. The purified XoxF enzyme contained La+3 suggesting that XoxF is important as a calcium-independent MDH that uses rare earth elements as cofactors (Nakagawa et al., 2012). In recently discovered methanotrophs of the phylum verrucomicrobia, xoxF is the only detectable gene that may code for MDH (Up de Camp et al., 2009). xoxF also occurs in non-methylotrophic bacteria, in which its metabolic function is unresolved (Chistoserdova et al., 2009). Thus, the detection of xoxF by NGS in environmental gene surveys or their occurrence in metagenomes, transcripts, and proteomes may be a hint to environmental methanol oxidation but need to be carefully evaluated based on recent and upcoming results from pure cultures of various organisms.

A comprehensive assessment of the genotypic diversity of aerobic methanol utilizers in the environment seems possible when mxaF, xoxF-like, mxaF2, mxaF, and genes of MDO of Actinobacteria are simultaneously analyzed. However, only mxaF has been successfully detected to date and PCR primers suitable for environmental surveys of the other genes have not yet been developed (McDonald and Murrell, 1997; Neufeld et al., 2007; Stacheter et al., 2013). More studies on the function of xoxF in further methylotrophs and addressing the physiological role of xoxF in organisms that are currently not known as methylotrophes are warranted to improve the ability to interpret methylotrophy gene-targeting surveys in the environment. The employment of
| Class/phylum                  | Isolation source | fac | pH | Reference                      |
|------------------------------|------------------|-----|----|--------------------------------|
| **Actinobacteria**           |                  |     |    |                                |
| Micrococcus luteus MM7        | Oral Cavity      | +   | n  | Anesti et al. (2005)           |
| **Bacilli**                  |                  |     |    |                                |
| Bacillus williamorita JY3A   | Soil             | +   | N  | Ling et al. (2011)             |
| **Alphaproteobacteria**      |                  |     |    |                                |
| Ancylobacter alcoholorumanicus DM11HT | Soil          | +   | N  | Firsova et al. (2009)          |
| Ancylobacter peterskowi N550 | Soil             | +   | N  | Lang et al. (2008)             |
| Ancylobacter polyornithus DSM 2467 | Soil          | +   | N  | Xin et al. (2006)              |
| Ancylobacter rudens.nih JCM 1167 | Rhizosphere  | +   | N  | Xin et al. (2004)              |
| Ancylobacter vacuolatus DSM 1277 | Soil           | +   | N  | Xin et al. (2006)              |
| **Betaproteobacteria**       |                  |     |    |                                |
| Methylobacillus arboreus VKM B-2590 | Phyllosphere  | +   | N  | Gogleva et al. (2011)          |
| **Gammaproteobacteria**      |                  |     |    |                                |
| Methylomonas koyamae MG30     | Water of rice paddy | –   | Act| Ogiso et al. (2012)            |
| Methylomonas scandinavica SRS | Groundwater      | –   | N  | Kalyuzhnaya et al. (1990)      |
| Methylomonas palpalis M30     | Torfmoor         | –   | Act| Danilova et al. (2013)         |
| Methylotethmus subterraneus DSM 19750 | Aquifer        | –   | Act| Hirayama et al. (2011)         |
| Methylotephalus lonarentis VPL | Sediment         | –   | Al | Antony et al. (2012)           |
| Methylotephalus sulfidovorans RB-1 | Sediment       | –   | N  | de Zwart et al. (1996)         |
| Methylotephalus thysoydanis DSM010 | Marine waters  | +   | n  | Boden et al. (2010)            |

fac, facultative methylotrophic; +, growth in substrates with carbon–carbon bonds; –, no growth in substrates with carbon–carbon bonds; n, no information in species description; enrichment at pH 7; Act, acer tolerant (growth optimum between pH 3.0 and 6.0); Ac, acacidiphilic (growth optimum below pH 3); Al, acalcaliphilic (growth optimum over pH 8).
genes of MDHs of Gram-positive methylotrophs will enhance the environmental detectability of methylotrophs and will aid to understand the role of these largely overlooked methylotrophs for environmental conversion of methanol has scarcely been studied (Table 1), and warrants, especially in terrestrial environments, future research.

POTENTIAL GENE MARKERS OF STRICT ANAEROBIC METHANOL UTILIZERS

The quantiative contribution of anaerobic methanol conversion in soils has scarcely been analyzed (Conrad and Claus, 2005). Beyond facultative aerobic methylotrophs, some strict anaerobes utilize methanol, i.e., methylotrophic methanogens and acetogens. A methanol-utilizing acetogen is Methanobrevibacter sp., and Methanomassiliococcus luminygensis. Among the strict anaerobic methylotrophic fungi, such as Candida, Pichia, in molds, and Ascomyctota (Table 1; Vandenbussche et al., 1992; Gousav et al., 2012). Known genes encoding for FAD AOs are mod1 and mod2, however homologs exist of which the function is unresolved (Nakagawa et al., 2006; Gousav et al., 2012). The use of genes of FAD AO for the environmental detection of methylotrophic fungi will be still challenging since numerous isoenzymes with likely different kinetic properties exist (Table 1; Ito et al., 2007). The role of the diversity and activity of fungal microorganisms for environmental conversion of methanol has scarcely been studied (Table 1), and warrants, especially in terrestrial environments, future research.

ASSESSMENT OF METHANOL UTILIZERS BY AMPLICON PYROSEQUENCING

The advent of NGS technologies allow for a dramatic increase of sequence information compared with similar efforts when using classic Sanger sequencing (Christen, 2008; Liu et al., 2012). The advent of NGS technologies allow for a dramatic increase of sequence information compared with similar efforts when using classic Sanger sequencing (Christen, 2008; Liu et al., 2012).
Table 3 | Gene markers of methanol-utilizing microorganisms for amplicon-based pyrosequencing or as targets for homology screens in metagenome, transcriptome, or proteome datasets.

| Methanol utilizers | Enzyme Function | Gene marker | Primers | Reference | Pyroseq |
|--------------------|----------------|-------------|---------|-----------|---------|
| Proteobacteria | PQQ MDH MeOH ox. | mxaF | 1003f/1555rE | McDonald and Murrell (1997); Neufeld et al. (2007) | Yes |
| Proteobacteria, Verrucomicrobia | Putative xoxF | mxaF | 1003f/1555rE | McDonald and Murrell (1997); Neufeld et al. (2007) | Yes |
| Burkholderiales | mxaF | mxaF | 1003f/1555rE | McDonald and Murrell (1997); Neufeld et al. (2007) | Yes |
| Various Proteobacteria, non-methylotrophs | Other | xoxF | fae1 | Kalyuzhnaya et al. (2004) | Yes |
| Actinobacteria | MCH | Other | mch-2a/mch-3 | Verholt et al. (1999); Kalyuzhnaya and Chistoserdova (2005) | Yes |
| Bacilli | Non available | MDO | mch | Park et al. (2010) | No |
| Methylotrophic methanogens | Methanol:CoM methyltransferase system | mtaC | Not available | Hagemeyer et al. (2008) | No |
| Methylotrophic acetogens | Methanol:CoM methyltransferase system-like | mtaC-like | Not available | Das et al. (2007) | No |
| Fungi | FAD AO | mod1, mod2, others | Not available | Red and Fewson (1994); Ozimek et al. (2005), Hartner and Glieder (2006); Nakagawa et al. (2006) | No |

MCH, methanol oxidation.

Primers for this group of genes have not been designed and tested in environmental surveys.

These enzymes do not oxidize methanol, but are involved in formaldehyde oxidation. These enzymes also occur in methylotrophs that do not use methanol and in non-methylotrophs (Chistoserdova, 2011).

Homologs of unknown function are present in methanogens (Ding et al., 2002).

Has been used in amplicon pyrosequencing.

Detect only mxaF and xoxF-like genes of Proteobacteria.

Amplicon pyrosequencing (i.e., a synonymous term is pyrotag sequencing) is one of the best evaluated and oldest NGS technologies (Liu et al., 2012). Long reads of about 700–1000 bp are possible, and technically unavoidable sequence errors can be removed with established software, such as AmpliconNoise (Quince et al., 2011; Rosen et al., 2012). Thus, large datasets with over 100,000 reads can be quality filtered, trimmed, and clustered into sequence similarity-defined operational taxonomic units (Caporaso et al., 2010; Nebel et al., 2011). Amplicon pyrosequencing comes along with higher costs per read compared to cheaper technologies, such as HiSeq, MiSeq, or Ion Torrent (Liu et al., 2012). However, the long-read length of pyrosequencing is especially advantageous when analyzing amplicons. Beyond that, amplicon-based pyrosequencing generates highly reproducible and similar community structures when compared to standard community fingerprinting techniques, such as terminal restriction fragment length polymorphism (TRFLP) analysis and thus, can be used for reliable genotype composition analyses (Pilloni et al., 2012).
the detection of xoxF-like genes (Table 3, Stacheter et al., 2013). When mxaF-targeting primers were used, also xoxF-like genes were detected in various grassland and forest soils by amplicon pyrosequencing (Stacheter et al., 2013) making the interpretation of data in regard to the capability of detected microorganisms of methanol oxidation more difficult, since the function of xoxF is still in part under debate.

**mxaF AND HOMOLOGS FOR ENVIRONMENTAL DETECTION OF METHYLOTROPHS**

Analysis of non-methanotrophic methylotrophs by mxaF genotyping has been employed in several studies. Nonetheless, only one study exist that employed amplicon pyrosequencing (Table 4).

All other amplicon-based NGS studies addressed methanotrophs and mostly analyzed pmoA (i.e., encodes a gene of a subunit of the particulate methane monoxygenase). The use of amplicon pyrosequencing is a great step forward toward complete coverage of the real diversity that exists in a given habitat. In this review, the authors argue in favor to target structural genes of methanol utilizers. One advantage of the use of structural genes is the increased sensitivity since rare groups, such as methylotrophs in soil communities, can be more reliably detected than by a 16S rRNA gene-based survey. Several methylotrophs occur in taxa of which only some members are capable of methylotrophy (e.g., Bacillaceae; Tables 1 and 2). The detection of such methylotrophs by 16S rRNA genes can be misleading, and thus, another advantage of the use of genes encoding a methanol-oxidizing enzyme is that the detection of the gene marker is linked with the potential phenotype of MUT. Nonetheless, gene marker-based phylogenies are not always congruent with organismal phylogenies (i.e., due to horizontal gene exchange between distantly related bacteria or evolution of functionally slightly different enzymes in the same organism; Friedrich, 2002). In general, mxaF-based phylogenies correlate with organismal phylogenies on the level of families of methylotrophs (Kist and Tate, 2013a,b; Lau et al., 2013). However, for other genes (mdh2, mda, mdh, mxaF, mxaF, methC) of methanol-oxidizing enzymes, congruence with organismal phylogenies needs to be evaluated.

Recent evaluation of phylogenetic resolution of mxaF compared to organismal phylogenies revealed contradicting results (Kist and Tate, 2013a,b; Lau et al., 2013). The congruency with the 16S rRNA gene phylogeny and the resolution of mxaF is sufficient (except for some “anomalies”) in the non-methanotrophic genus *Methylobacterium* (affiliates with Alphaproteobacteria), i.e., the so-called pink-pigmented, facultatively methylotrophic (PFPM) bacteria (Kist and Tate, 2013a,b), and mxaF-based taxonomic resolution might be even higher than that of 16S rRNA genes (Kist and Tate, 2013b). Nonetheless, strain-level identification is not possible and requires the analysis of more variable genomic regions (Kist and Tate, 2008, 2010b). Some alpha-proteobacterial genera harbor mxaF-like genes that are similar to those of *Methylobacterium* suggesting the occurrence of horizontal gene transfer events in evolution of methylotrophs; *Methylobacterium nodulans* ORS 2860A carries a plasmid with methylotrophy genes including mxaF, suggesting that this species has acquired this gene from another PFPM bacterium (Kist and Tate, 2013a). Using mxaF as a phylogenetic marker of methanotrophic Proteobacteria revealed that the three major families *Methylcocccaceae, Methylocystaceae*, and *Beijerinckiacae* can be unambiguously reconstructed (Lau et al., 2013). Nonetheless, mxaF and 16S rRNA gene phylogenies differ on genus and species level (Lau et al., 2013). The loose coupling of mxaF phylogenies with 16S rRNA gene phylogenies is reflected by a low DNA-level similarity (about 77%) that relates to a 97% similarity cut-off on 16S rRNA gene sequence level, which is indicative for species. This low mxaF cut-off level even decreases when more species are considered (Stacheter et al., 2013) supporting the conclusion that horizontal gene transfer occurred between methylotrophs and non-methylotrophs. A frequently detected

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**Table 4 | Use of amplicon pyrosequencing to analyze methylotrophic communities.**

| Environment | Gene markers | Remarks | Functional group | Reference |
|-------------|--------------|---------|-----------------|-----------|
| **Soils**   |              |         |                 |           |
| Aerated soils | mxaF, rnh, faeI | Amplification with adapter-less primers | Bacterial methylotrophs | Stacheter et al. (2013) |
| Hydromorphic grassland soil | pmoA | – | Methanotrophs | Stacheter et al. (2013) |
| Peatland | pmoA | – | Methanotrophs | Deng et al. (2013) |
| Peat bog | pmoA | Read length > 500 nt, blended analysis with other genes | Methanotrophs | Kim et al. (2013) |
| **Aquatic habitats** | | | | |
| Lake sediments and waters | 16S rRNA genes | Combined with DNA SIP | Methanotrophs | Ha et al. (2012) |
| Water of oil sand tailings ponds | pmoA | – | Methanotrophs | Saidi-Mehrabad et al. (2013) |
| **Aquifer** | | | | |
| Aquifer | 16S rRNA genes | V4-V6 region of 16S rRNA | Methanotrophs and others | Lavalleur and Colwell (2013) |
| **Technical systems** | | | | |
| Methanotrophic biotopes | 16S rRNA genes | – | Methanotrophs and others | Kim et al. (2013) |
**REFERENCES**

Anesi, V., Mokrond, I. R., Kumaravanti, M., Wade, W. G., Kelly, D. P., and Wood, A. P. (2005). Isolation and molecular detection of methylothrophic bacteria occurring in the human mouth. Environ. Microbiol. 7, 1227–1230. doi:10.1111/j.1462-2920.2005.00690.a

Angel, R., and Conrad, R. (2009). A comparison of pyrosequencing and hybridization-based methods for the identification of novel NADP(H)-containing methanolic dehydrogenase active methanol utilizers in terrestrial and other environments, and may allow for detection of novel oxygenreductases and microorganisms that utilize methanol in the environment. Still not resolved issues for a comprehensive detection of methanotrophs are (a) the unresolved quantitative impact of organisms that employ methanol-oxidizing enzymes than methanol-specific oxidoreductases, and (b) the detection of methanotrophs that disintegrate but do not assimilate methanol-derived carbon. Such organisms might be detectable by SIP when using their actual carbon substrate as a source of isotopic label combined with unlabelled methanol and a control, in which the labelled substrate is not substitute.

Bambhorst, C. W., and Querleu, J. R. (1978). Aerobic and anaerobic degradation of benzenoic acid on methanol. Arch. Microbiol. 119, 91–97. doi:10.1007/BF00407924

Berry, D., Ben Mahfoudh, K., Wagner, O., Neumann, B., and Lepper, M. (2010). Amplified primers used in multiplex amplicon pyrosequencing and bioinformatic analysis. Appl. Environ. Microbiol. 77, 7846–7849. doi:10.1128/AEM.01790-10

Basett, A., Abdel, G. C. I., Reddy, L., Richardson, A. E., and Thrall, P. H. (2012). Methanotroph communities in Australian woodland soils of varying salinity. FEMS Microbiol. Ecol. 80, 685–695. doi:10.1111/j.1574-6941.2012.01541.x

Boden, R., Kelly, D. P., Murlill, J. C., and Schäfer, H. (2009). Oxidation of dimethylfumarate to tetrahydrofurfuryl alcohol is involved in the C1 metabolism of the Gram-positive methylotrophic bacterium Paracoccus denitrificans sp. nov. as a new link in the salinity cycle. Environ. Microbiol. 12, 2603–2622. doi:10.1111/j.1462-2920.2010.02258.x

Brotherton, L. V., Antronova, L. R., and Antonova, Y. A. (1998). Methanol metabolism in mutants of the methylo trophic yeast Hansenula polymorpha. FEMS Microbiol. Lett. 51, 89–93. doi:10.1111/j.1574-6960.1988.tb02737.x

Brotherton, L. V., Ushakovich, J., Vanbruggen, E. F. J. M., Fan, J., Sany, B., Nikiforuk, N. I., et al. (1995). Electron microscopic analysis and structural characterization of novel NADP(H)-containing methanol–N,N-dimethyl-4nitrosoaniline oxidoreductase from the Gram-positive methylo trophic bacterium Arthromyces sp. MB10. J. Bacteriol. 177, 1814–1822.

Cavarsan, J. G., Kalyuzhnaya, I., Stombaugh, J., Rüttinger, K., Budman, F. D., Conklin, E. K., et al. (2010). QIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 555–556. doi:10.1038/nmeth.f130

Chistoserdova, L. (2011). Modulation of methylothrophy, revisited. Environ. Microbiol. 13, 2603–2622. doi:10.1111/j.1462-2920.2010.02260.x

Chistoserdova, L., Kalyuzhnaya, M. G., and Lipton, M. E. (2009). The expanding world of methylo trophic metabolism. Annu. Rev. Microbiol. 63, 477–495. doi:10.1146/annurev.micro.091208.073600

Christen, R. (2008). Global sequencing: a review of current molecular data and new methods available to assess microbial diversity. Microb. Ecol. 57, 255–268. doi:10.1007/s00248-008-9402-5

Das, A., Xu, Z. Q., Tukala, W., Liu, Z. J., Chang, J., Chen, L. R., et al. (2007). Characterization of a carnitine positive Methylococcus capsulatus consortia in Riganqiao peatlands. Microb. Ecol. 53, 167–176. doi:10.1007/s00248-007-9104-0

Deufyl, S. N. (2008). Exploring methylo trophic diversity in acidic northern soils: molecular and cultivation-based studies. Microbiology 78, 655–669. doi:10.1099/mic.0.20758-0

Delmonte, N., Reif, C., Chaf- fron, S., Imslandb, G., Roeschlin, B., Schlapbach, R., et al. (2009). Community proteogenomics reveals insights into the physiology of phylophore bacteria. Proc. Natl. Acad. Sci. U.S.A. 106, 16428–16433. doi:10.1073/pnas.0912521106

Deng, Y., Xi, X., Liu, L., and Dumont, M. G. (2013). Aerobic methanotrophic diversity in Beggiatoa portlandii on the Qinghai-Tibetan plateau. Environ. Microbiol. Rep. 5, 566–574. doi:10.1111/1758-2229.12046

Dovgan, G. E., Aitken, N., Top- stra, P., and Dijkhuizen, L. (1992).
Ding, Y. H. R., Zhang, S. P., and Tomb, J. (2003). Wilson and characterization of Methylocystis ethaneoxidans sp. nov., an obligately methylotrophic, aromatic, dimethylsulfide oxidizing bacterium from microalgal mat. FEMS Microbiol. Ecol. 48, 261–270. doi: 10.1111/j.1574-6941.1999.480043.x

Ding, Y. H. R., Zhang, S. P., and Tomb, J. (2003). Methanotrophic commensal methanotrophs from rice rhizosphere. FEMS Microbiol. Lett. 215, 127–132. doi: 10.1111/j.1574-6968.2003.tb07834.x

Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D. W., Haywood, J., Myhre, G., Nas Gleckner, J. H., lnkelhinn, A., and Leck, I. D. (2007). Climate change 2007: The physical science basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change, ed S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. A. Averyt, et al. (Cambridge: Cambridge University Press), 12–234.

Frech, M. W. (2002). Phylogenetic analysis reveals multiple lateral transfers of adenyls-5'-phosphate: reductase genes among sulfate-reducing microorganisms. J. Bacteriol. 184, 278–289. doi: 10.1128/JB.184.1.278-289.2002

Galabry, E. L. and Kristin, W. (2002). The production of methanol by flowering plants and the global cycle of methanol. J. Atmos. Chem. 43, 195–229. doi: 10.1021/PA020684Y

Givargis, V. P., Hara, D., A. H., Tripp, H. J., Smitl, U., Gm, A. S., Cho, J. C., et al. (2008). The small genome of an abundant coastal ocean methylophile. Environ. Microbiol. 10, 1711–1722. doi: 10.1111/j.1462-2920.2008.01584.x

Hirama, H., Suna, K., Abu, M., Miyazaki, M., Makita, H., Inagaki, F., et al. (2011). Methylophilus okinawanus sp. nov., a moderately thermophilic methanotroph isolated from a terrestrial subsurface hot aquifer. Int. J. Syst. Evol. Microbiol. 61, 2646–2653. doi: 10.1099/ijs.0.038628-0

Holmes, A. J., Constable, A., Lutken, M. E., and Murrell, J. C. (1995). Evidence that particular methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol. Lett. 125, 205–208. doi: 10.1111/j.1574-6941.1995.00510.x

Huang, T., Peters, K., and Schmidt, E. (2011). Methylocellobacter illuviation sp. nov., a methylophilic bacterium isolated from Fumana hypermuscetous Syn. Appl. Microbiol. 54, 462–469. doi: 10.1016/j.syapm.2010.12.005

Idris, R., Kuffner, M., Bodrossy, L., Puschmann, M., Manthel, S., Wemml, W., et al. (2006). Characterization of a Ni-tolerant methylotroph isolated from the hyperaccumulating plant Thelypteris geopon and description of Methylobacterium geopon sp. nov. Syn. Appl. Microbiol. 32, 638–644. doi: 10.1016/j.syapm.2006.01.011

Im, W. T., Adam, Z., Lee, M., Ten, L. N., Yang, D. C., and Lee, S. T. (2008). Starkeya koreensis sp. nov., isolated from rice straw. Int. J. Syst. Evol. Microbiol. 58, 2409–2414. doi: 10.1099/ijs.0.01489-0

Inoue, T., Fujimura, S., Ichino, M., Tanaka, N., Matsuishi, Y., Misri, T., et al. (2007). Distribution, diversity and regulation of alcohol oxidase isozymes, and phylogenetic relationships of methylotrophic yeasts. FEMS Yeast Res. 8, 325–332. doi: 10.1111/j.1567-1364.2007.00159.x

Jacob, D. J., Field, B. D., L. B., Blake, D. R., De Gouw, J., Warneke, C., et al. (2005). Global budget of methanol constraints from atmospheric observations. J. Geophys. Res. Atmos. 110, D09303. doi: 10.1029/2004JD005172

Kolb and Stacheter Pyrosequencing of environmental methanotrophic utilizers
isotope probing. Nat. Prod. 5, 1937–
1966. doi: 10.1038/np.2010.186
Kolb, N. D., Deliš, B. E., Pan, K.,
Byrdon, L., Neveling, K., Kortel,
M., et al. (2011). Ultra-deep
pyrosequencing of pmoA amplicons
from anoxic marine sediments. Appl.
Environ. Microbiol. 77, 6865–6871.
doi: 10.1128/AEM.01573-11
Kolb, N. D., and Fennell, P. (2011).
Potential of pmoA amplicon
pyrosequencing for methanotroph
diversity studies. Appl. Environ.
Microbiol. 77, 6805–6809.
doi: 10.1128/AEM.05975-11
Li, C., Krause, S., Cartegno,
S., Greppi, D., Lutteke, R.,
and Frenzel, P. (2010).
Biogeography of wetland rice
methanotrophs. Environ. Microbiol. 12,
862–872. doi: 10.1111/j.1462-2920.
2009.01935.x
Madhavan, M., Poonguzhali, S.,
Kwon, S. W., and Sa, T. M. (2006).
Methylobacterium phyllosphaerae sp.
nov., a pink-pigmented, facultative
methanotroph from the phyllosphere of
rice. Int. J. Syst. Evol. Microbiol. 56,
22–27. doi: 10.1099/ijs.0.65691-0
McDonald, I. K., and Murrell, J. C.
(1997). The methyl-6-deoxy-
6-methyl-hexose structural gene and its
use as a functional gene probe for
methanotrophs and methanomethy-
lotes. Appl. Environ. Microbiol. 63,
5328–5334
Moshiri, S. A., Pacheco, C. C.,
Mulock, I. R., De Marco, L., Parry,
A. D., Kelly, D. P., et al. (2005).
Isolation and properties of methanol-ad-
degradating Agrobacterium sp. from
Antarctica and comparison with other
strains of A. vitis. Environ. Microbiol.
7, 23–35. doi: 10.1111/j.1462-2920.
2004.00664.x
Mustakirimin, I., Kolb, N. D.,
Fennell, P., and Chistoserdova,
L. (2013). In situ den-
itrification in Methylomonas mobile
denitrification pathway and methyl-
ated methanogenesis pathways. J.
Environ. Monit. 15, 2007–2011.
doi: 10.1039/c3em20089a
Nagakawa, T., Inagaki, A., Ino,
T., Fushimi, S., Maruyama,
H., et al. (2006). Regulation of two
distinct alcohol dehydrogenase
promoters
Kolb and Stacheter
Pyrosequencing of environmental methanotrophs
in the methylo trophic yeast Pichia methanolica. Yeast 23, 15–22. doi: 10.1007/s12328-012-9584-6
Nakagawa, T., Mitani, R., Tani, A., Suga, K., Takeda, S., Samei, T., et al. (2012). A catalytic role of
XoxF in L-α-ketoacid-dependent methanol dehydrogenase in Methylobacterium extorquens strain AM1. PLoS ONE 7:e38036. doi: 10.1371/journal.pone.0038036
Nakahara, T., Uehara, T., and Komagata, K. (1996). Isolation of methanol oxidase in a methane-utilizing
yeast, Pichia methanolica IAM 12901. J. Ferment. Bioeng. 83, 408–409. doi: 10.1006/jfbi.1996.10781
Nebel, M. E., Wild, S., Holzhauser, O., Ogiso, T., Ueno, C., Dianou, D., Huy, T., et al. (2013). Kolb and Stacheter Pyrosequencing of environmental methanol utilizers.
Neufeld, J. D., Schäfer, H., Cox, M. Nozaki, M., Suzuki, N., and Washizu, N. (2008). Applications of pyrosequencing to environmental metanol utilizers. PLoS ONE 3:e3818. doi: 10.1371/journal.pone.0003818
Nebel, M. E., Wild, S., Holzhauser, O., Ogiso, T., Ueno, C., Dianou, D., Huy, T., et al. (2013). Kolb and Stacheter Pyrosequencing of environmental methanol utilizers.
Nebel, M. E., Wild, S., Holzhauser, O., Ogiso, T., Ueno, C., Dianou, D., Huy, T., et al. (2013). Kolb and Stacheter Pyrosequencing of environmental methanol utilizers.
Nebel, M. E., Wild, S., Holzhauser, O., Ogiso, T., Ueno, C., Dianou, D., Huy, T., et al. (2013). Kolb and Stacheter Pyrosequencing of environmental methanol utilizers.
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