Application of Omics Approaches to Studying Methylotrophs and Methylotroph Communities

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Abstract

This review covers some recent advances in application of omics technologies to studying methylotrophs, with special reference to their activities in natural environments. Some of the developments highlighted in this review are the new outlook at the role of the XoxF-type, lanthanide-dependent methanol dehydrogenase in natural habitats, new mechanistic details of methane oxidation through the reverse methanogenesis pathway, propensity of 'aerobic' methanotrophs to thrive in hypoxic environments and potential connection of this process to denitrification, and a novel outlook at methane oxidation as a community function.

Introduction and overview

Metabolism of single-carbon (C1) compounds, i.e. compounds not containing any carbon–carbon bonds, such as methane and other methylated compounds, is a significant part of the global carbon cycle. Microbes require specialized biochemical pathways and thus specialized genetic machineries in order to extract energy from methylated compounds and also to build biomass from C1 units such as formaldehyde (heterotrophic methylotrophy), CO_2 (autotrophic methylotrophy) or a combination. The ability to gain both energy and carbon from C1 compounds makes true methylotrophs (Anthony, 1982; Chistoserdova and Lidstrom, 2013). However, organisms can also separate C1-based energy metabolism from carbon assimilation, to follow a myxotrophic metabolic mode (Chistoserdova, 2011). These have been termed 'methylovores' (Sun *et al*., 2011). While important players in environmental cycling of carbon, methylotrophs also play roles in global nitrogen, sulfur and phosphorous cycles (Chistoserdova, 2015). One of the most abundant C1 compounds in the environment is methane that is produced both abiogenically, through processes in the Earth's crust (Sherwood Lollar *et al*., 2002; 2006) and biogenically, through microbial

methanogenesis (Singh *et al.*, 2010). Other abundant C1 compounds are methanol, naturally generated by plants (Galbally and Kristine, 2002; Jacob *et al.*, 2005; Mincer and Aicher, 2016), methylated amines generated in aquatic environments (Naqvi *et al.*, 2005; Lidbury *et al.*, 2015), halogenated methanes, occurring naturally as well as through human activities (Muller *et al.*, 2011), and methylated sulfur species such as dimethylsulfide (DMS) and dimethylsulfoxide (DMSO), produced in marine environments (Watts, 2000).

As a metabolic trait, methylotrophy has been known since the early twentieth century, when bacteria capable of growth on methane were first characterized (Kaserer, 1906; Söhngen, 1906). Since then, methylotrophic species have been characterized belonging to Alpha-, Beta-, and Gammaproteobacteria, Actinobacteria, Firmicutes, Verrucomicrobia and a Candidate phylum NC10 (Chistoserdova and Lidstrom, 2013). Specific lineages within the Archaea have also been shown to be active in methane oxidation (Knittel and Boetius, 2009; Haroon *et al.*, 2013; Offre *et al.*, 2013). However, likely, methylotrophy or methylovory are widespread beyond these taxa and across the tree of life, even if their representatives avoided characterization so far.

The biochemistry of methylotrophy involves specific enzymes and pathways for oxidation and assimilation of C1 substrates, which are rather well understood, at least when it comes to the cultivated methylotrophs (Anthony, 1982; Chistoserdova, 2011; Chistoserdova and Lidstrom, 2013). In general, each C1 substrate requires a dedicated enzyme to convert it into a less reduced C1 compound, and these are considered primary C1 oxidation modules. In Bacteria, these include two distinct types of methane monooxygenases, soluble (sMMO) and particulate (pMMO; Sirajuddin and Rosenzweig, 2015), two distinct methanol dehydrogenases (MDH), MxaFI and XoxF (Anthony, 2004; Williams *et al.*, 2005; Pol *et al.*, 2014; Chistoserdova, 2016), a methylamine dehydrogenase (MADH; Davidson, 2003, 2004) and an alternative, multienzyme pathway for methylamine oxidation (Chen *et al.*, 2010; Latypova *et al.*, 2010). Specific primary oxidation modules are also dedicated to metabolism of methylated halogen and sulfur species (Muller *et al.*, 2011; Lidbury *et al.*, 2015, 2016).

Oxidation of the primary C1 substrates results in a common currency of methylotrophy, formaldehyde, methylene tetrahydrofolate (H_4F), formate or CO_2. At this point methylotrophs still have a number of choices for how to combine the multiple modules for oxidation and assimilation of these compounds (Chistoserdova, 2011; Chistoserdova and Lidstrom, 2013).

Organisms that have served as models for experimental validation of the details of the biochemical pathways enabling methylotrophy have been referred to as 'aerobic' and sometimes 'strictly aerobic' as none of them grew in the absence of oxygen (Trotsenko and Murrell, 2008), acetogenic clostridia being one exception (Ragsdale and Pierce, 2008). Reactions such as methane oxidation are indeed strictly oxygen-dependent (Sirajuddin and Rosenzweig, 2015). However, 'anaerobic' methane oxidizers have been identified that operate some of these pathways, including methane oxidation, (Ettwig *et al.*, 2010). To resolve this paradox, a novel enzyme has been proposed that would make oxygen intracellularly by dismutation of nitrous oxide (Ettwig *et al.*, 2010). However, existence of such an enzyme has not been proven biochemically so far.

The term 'methylotrophic archaea' has been used for a long time, and originally it referred to methanogens that are capable of converting methylated compounds, such as methanol and methylated amines, into methane through methyl transfer (Costa and Leigh, 2014).

However, more recently, archaea very closely related to the classic methanogens have emerged as methylotrophs that can actually oxidize methane by reversing the methanogenesis pathway, and these became known as 'anaerobic methane oxidizers' (ANME, Knittel and Boetius, 2009). In this case, another paradox persisted for decades (reviewed by Valentine and Reeburgh, 2000), the problem of methane activation in the absence of oxygen. This paradox has been recently resolved by experimental demonstration of reverse reaction activity for methyl-CoM reductase (MCR; Scheller *et al*., 2010) and by demonstration of striking structural similarities between the MCR enzymes involved in methane generation and methane oxidation (Shima *et al*., 2011).

While biochemically, methylotrophy in bacteria is quite different from methylotrophy in Archaea, the two processes share at least some biochemistry in common. For example, C1 transfer reactions linked to methanopterin and methanofuran derivatives are common in both (Fig. 7.1), and the reactions of the Wood–Lungdal pathway are also found in both Archaea and Bacteria, suggesting both interesting evolutionary scenarios (Braakman and Smith, 2000) and potential existence of further combinations of metabolic pathways that carry out novelty types of methylotrophy.

As any other subfield of Biology, in recent years the methylotrophy field has relied on the omics technologies to study the diversity of methylotrophs, their presence and abundance in a variety of environmental niches, their specific activities in situ as well as the interspecies interactions. As omics technologies gradually became more high throughput and more affordable, their applications expanded from sequencing amplified gene products

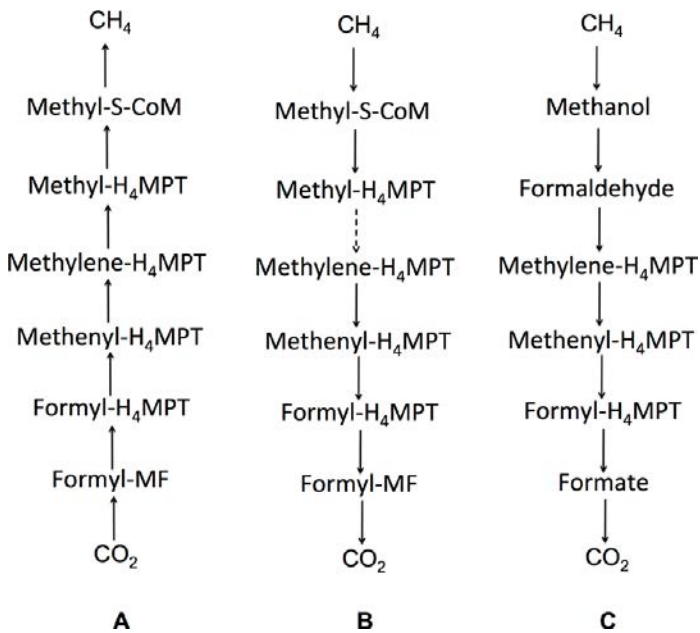

Figure 7.1 The commonality of pathways for methanogenesis and methane oxidation. A, methanogenesis; B, methane oxidation by archaea; C, methane oxidation by bacteria. H_4MPT, tetrahydromethanopterin; MF, methanofuran. For details of reactions and enzymes, see Thauer, 2011; Chistoserdova *et al.*, 2009.

for environmental detection, to sequencing genomes and transcriptomes, to whole genome shotgun sequencing metagenomics, metatranscriptomics and metaproteomics (Chistoserdova, 2015). These and other systems approaches are enabling further advances in understanding methylotrophs and methylotroph communities in the environment. In this review I cover some recent discoveries through application of systems biology approaches that shift several long-term paradigms in methylotrophy, originally formed based on studies in laboratory models. These discoveries expand our knowledge of methylotrophy and its distribution and evolution as a metabolic phenomenon. The recent discoveries, in turn, create new paradigms. These new paradigms point to fewer juxtapositions between archaeal and bacterial metabolisms, between aerobic and anaerobic metabolic modes, and they also highlight metabolic synergy among different microbial guilds in utilization of C1 compounds.

New insights into methanol oxidation

The *Methylotenera* paradox and (re)discovery of a novel methanol dehydrogenase

MDH consisting of two types of subunits, MxaFI is one of the most well studied enzymes in methylotrophs, and, in pre-genomic studies, it has been found in all model methylotrophs that grew on methanol (Anthony, 1982, 2004; Williams *et al*., 2005). About two decades ago, a gene has been identified, *xoxF*, whose product XoxF showed approximately 50% amino acid sequence identity with MxaF (Chistoserdova, 1996; Harms *et al*., 1996). However, no MDH activity could be measured for this protein, and mutation in *xoxF* did not affect growth on methanol (Chistoserdova and Lidstrom, 1997), and its role remained a mystery for years (Chistoserdova, 2011).

One of the early metagenomics studies as applied to methylotrophs has utilized stable isotope probing (DNA-SIP) to label species actively consuming methylated compounds in lake sediment, using microcosm incubations (Kalyuzhnaya *et al*., 2008). This study has shown that *Methylobacter* type methanotrophs were the dominant active species, in contrast with data from cultivation approaches from the same study site that identified *Methylomonas* and *Methylosinus* instead (Auman *et al*., 2000). Metabolic reconstruction from the composite *Methylobacter* genome assembled in this study identified all the expected methylotrophy pathways, but also identified genes for respiratory denitrification, suggesting one alternative pathway to oxygen respiration.

Somewhat less expected, highly assembled composite genomes were also recovered in this study for a then novel species of the *Methylophilaceae* family, *Methylotenera,* that are unable to oxidize methane (Kalyuzhnaya *et al*., 2006, 2012). *Methylotenera* genomes were also assembled from methylamine- and methanol-fed microcosms. The *Methylotenera* phylotypes from the methylamine microcosm encoded functions for MADH, in agreement with their success in consuming methylamine. However, none of the *Methylotenera* genomes appeared to encode a methanol dehydrogenase (MxaFI), until then readily identified in all known *Methylophilaceae* (Doronina *et al*., 2004, 2005a,b). This created a *Methylotenera* paradox: first, how did they consume methanol (in the methanol microcosm); second, how did they obtain labelled carbon from methane (in the methane microcosm)? An additional question that remains unanswered is why other methylotrophs that could be

easily cultivated from the same study site (Beck *et al.*, 2015) were not competitive against *Methylotenera* species? Interestingly, from metagenome-based metabolic reconstruction, some of the *Methylotenera* phylotypes also encoded respiratory denitrification functions (Beck *et al.*, 2014). Further DNA-SIP experiments with labelled methanol uncovered that different *Methylotenera* phylotypes revealed preferences for different nitrogen sources (Kalyuzhnaya *et al.*, 2009), further suggesting denitrification capabilities, which were ultimately demonstrated through nitrogen species measurements in pure cultures and through mutant analysis (Kalyuzhnaya *et al.* 2009; Mustakhimov *et al.*, 2013). The genes/proteins potentially involved in metabolism of methanol by *Methylotenera* species were assessed through transcriptomics and proteomics, and both approaches identified as potential candidates XoxF proteins (Bosch *et al.*, 2009; Vorobev *et al.*, 2013). Moreover, mutation of *xoxF* genes produced a methanol-negative phenotype (Mustakhimov *et al.*, 2013). These data have solved the *Methylotenera*/methanol paradox, while they further reinforced the XoxF paradox (Bosch *et al.*, 2009; Vorobev *et al.*, 2013; Mustakhimov *et al.*, 2013).

XoxF is a major methylotrophy enzyme, and it requires lanthanides for activity

The XoxF paradox was finally resolved by a demonstration of its requirement for lanthanides (Ln^{3+}) for MDH activity (Hibi *et al.*, 2011; Fitriyanto *et al.*, 2011; Nakagawa *et al.*, 2012; Pol *et al.*, 2014). More recent studies demonstrated that not only this Ln^{3+}-dependent enzyme can carry out methanol oxidation, but that it is an essential component of methanol utilization by both methanol- and methane-utilizing bacteria (Haque *et al.*, 2015; Vu *et al.*, 2016; Chu and Lidstrom, 2016). These findings have overturned the long-held assumption that the dominant methanol-oxidizing enzyme in nature is the calcium-dependent, MxaFI-type MDH. In addition, these new discoveries demonstrated the existence of Ln^{3+}-dependent enzymes, overturning prior assumptions that Ln^{3+} are inert with respect to the biochemistry of living organisms (Lim and Franklin, 2004). Moreover, it was demonstrated that not only were Ln^{3+} required for XoxF-MDH activity, but they were also involved in regulation of both *xox* and *mxa* gene clusters acting as a regulatory switch between the two MDH enzymes (Haque *et al.*, 2015; Chu and Lidstrom, 2016; Vu *et al.*, 2016). The fact of nanomolar amounts of Ln^{3+} being sufficient to activate XoxF expression suggested active transport of Ln^{3+} into the cell (Pol *et al.*, 2014; Vu *et al.*, 2015; Gu *et al.*, 2016).

A crystal structure has been obtained for one Ln^{3+}-dependent MDH, from *Methylacidiphilum fumariolicum* (Pol *et al.*, 2014). Its analysis identified the likely ligands for binding Ln^{3+} ions, two of which were conserved with respect to those binding Ca^{2+} in the active centre of MxaFI-type MDH enzymes, while an additional ligand, specific to XoxF enzymes, was identified and proposed to be specific for accommodating the trivalent Ln^{3+}(Pol *et al.*, 2014).

Along with the biochemical evidence, support for the environmentally important function of the Ln^{3+}-dependent enzymes has been accumulated, mostly through-omics studies conducted in natural or in simulated environments. This evidence points at XoxF activity in some major biogeochemical cycles, based on estimates of its abundance and expression in specific niches, such as methane-consuming layers of lake sediments and mud pots, marine and freshwater columns, phyllo- and rhizosphere. These data have been recently summarized by Chistoserdova (2016), and examples are presented in Table 7.1.

Likely, these findings merely mark the very beginning of appreciation of Ln^{3+}-dependent

Table 7.1 XoxF detection in environmental or manipulated samples

Environment	Organism	Method	Reference
Biphenyl and benzoate-degrading laboratory culture	*Burkholderia xenovorans*	Transcriptomics, proteomics	Denef *et al*., 2004, 2005
Lake sediment methylotrophic community	Functional metagenome enriched in *Methylobacter*, *Methylotenera*	Metagenomics	Kalyuzhnaya *et al*., 2008; Beck *et al*., 2013
Lake sediment methylotrophs	Lake sediment community, *Methylotenera mobilis*, *Methylotenera versatilis*	Microarray hybridization, transcriptomics	Kalyuzhnaya *et al*., 2010; Vorobev *et al*., 2013
Plant phyllosphere	*Methylobacterium*	Metaproteogenomics	Delmotte *et al*., 2009
Coastal ocean waters	*Methylophilaceae*	Metaproteomics	Sowell *et al*., 2011
Coastal ocean waters	*Methylophaga*	Stable isotope probing/proteomics	Grob *et al*. 2015
Coastal marine environments	*Rhodobacteraceae*, *Methylophilaceae*	PCR amplification	Taubert *et al*., 2015
Plume waters off a hydrothermal vent	Bacteria	Metaproteomics	Mattes *et al*., 2013
Sea sponge	Sea sponge symbiont	Metatranscriptomics	Moitinho-Silva *et al*., 2014
Marine, estuarine and freshwater environments	*Methylophilaceae*	Quantitative PCR	Ramachandran and Walsh, 2015

biochemistry as related to the functionality of organisms inhabiting important environmental niches on this planet. These recent developments highlight some significant gaps in understanding of the details of biochemistry and regulation of methylotrophy in environmentally important microbes, the role of metals such as Ln^{3+} in the biochemistry if living organisms, as well as the potential role of Ln^{3+} as drivers of biogeochemical cycles (Chistoserdova, 2016).

New insights into methane oxidation by archaea

Methane oxidation through reverse methanogenesis linked to sulfate reduction

Geochemical evidence existed for a long time suggesting anaerobic methane oxidation linked to sulfate reduction (reviewed by Valentine and Reeburgh, 2000). However, the process remained enigmatic for decades, based on the very unfavourable bioenergetics of such a process (Thauer, 2011). However, molecular evidence was generated circa 2000, identifying Euryarchaeota archaea related to bona fide methanogens as organisms (ANME) capable of anaerobic oxidation of methane (AOM), in syntrophy with sulfate reducing bacteria (SRB; Hinrichs *et al*., 1999; Boetius *et al*., 2000; Orphan *et al*., 2001). The genomic

details potentially explaining the mechanism for their interaction could only be obtained through metagenomics as none of these organisms are available in culture. Hallam *et al.* (2004) undertook one of the early metagenomic sequencing efforts, isolating DNA from anoxic sediment in California with high measured rates of AOM, enriched in AMNE-1 type archaea. Sequence analysis has revealed the presence of most of the genes for methanogenesis, with one exception (*mer,* encoding methenyltetrahydromethanopterin reductase was missing), providing support to the reverse methanogenesis hypothesis (Hallam *et al.*, 2004). Similar results were obtained when metagenomic sequencing was carried out with samples from the Black Sea (Meyerdierks *et al.*, 2010). In this study, a nearly complete ANME-1 archaeon genome was assembled and analysed, supporting the findings by Hallam *et al.* (2004). Moreover, expression of the methanogenesis genes was also proven through mRNA sequencing. In this study as well, the genes for Mer could not be identified, but a bypass pathway was proposed (Meyerdierks *et al.*, 2010). Interestingly, both genomic and transcriptomic analysis in this study have identified a potential for direct electron transfer between the syntrophic partners, via multiheme cytochromes (Meyerdierks *et al.*, 2010). A metaproteogenomic analysis of a cold seep mat community enriched in ANME-1 organisms provided further evidence for the methanogenesis pathway being active, again not detecting Mer (Stokke *et al.*, 2012). These metagenomic data were all in agreement with previous data on detection of highly abundant proteins in ANME-dominated microbial mats, identified as subunits of methyl-CoM reductase (MCR; Krüger *et al.*, 2003), even though the fate of methyl group immediately after methane oxidation could not be fully resolved.

In a more recent study, Wang *et al.* (2014) assembled a highly covered genome of an ANME-2 archaeon, from highly active laboratory enrichment, and mapped transcript reads from the same enrichment to this genome. In this case, the complete methanogenesis pathway was reconstructed, and all the relevant genes were expressed (Wang *et al.*, 2014). The metagenomic studies, lending strong support for reverse methanogenesis, have facilitated further progress in understanding the process of AOM, including mechanisms for methane activation and for interspecies electron transfer. Towards validating the involvement of MCR in methane activation, reverse reaction activity for MCR from a bona fide methanogen has been experimentally demonstrated (Scheller *et al.*, 2010). Additional validation was obtained through purification and crystallization of an MCR homologue from ANME-1-enriched microbial mat (Shima *et al.*, 2011). This enzyme revealed striking structural similarities with MCR enzymes involved in methanogenesis (Shima *et al.*, 2011), further suggesting that methane production and methane oxidation may rely on the same enzyme acting in reverse. Indeed, Lloyd *et al.* (2011) have demonstrated that ANME organisms can both produce and oxidize methane. Their conclusions were based on quantifying gene transcripts of ANME in zones of methane oxidation and methane production, separated across the depths of sediment (Lloyd *et al.*, 2011).

Towards resolving the long-standing problem of interspecies electron transfer, direct coupling of AOM to sulfate reduction by ANME was proposed (Milucka *et al.*, 2012). However, this proposal contradicted the well-documented co-occurrence and tight physical association of ANME and SRB (Boetius *et al.*, 2000; Orphan *et al.*, 2001). The hypothesis of direct electron transfer (DIET) between ANME and SRB was further supported by data from several recent studies. Wegener *et al.* (2015) concluded on the probability of DIET based on analysis of the transcriptomes generated for syntrophic communities of ANME and SRB active in thermophilic AOM. Key to this study was the possibility to manipulate

the SRB partner in the absence of the ANME partner (Krukenberg *et al.*, 2016), demonstrating that functions such as sulfate reduction, pili formation and multiheme cytochrome synthesis were up-regulated in SRB cultures growing syntrophically compared to SRB cultures growing on hydrogen. Moreover, nanowire-like structures were detected connecting ANME and SRB cells, resembling the structures previously characterized in *Geobacter* consortia (Wegener *et al.*, 2015). In a separate study, a similar conclusion was reached through spatially resolved analysis of cellular activities in ANME-SRB consortia, metagenomics and microscopy studies (McGlynn *et al.*, 2015). Further support for DIET was obtained through decoupling AOM from sulfate reduction through the use of artificial electron acceptors (Scheller *et al.*, 2016). Respiration with solid electron acceptors via extracellular metal reduction would be one mechanism responsible for the described AOM coupled to insoluble iron (III) and manganese (IV) (Beal *et al.*, 2009).

Methane oxidation linked to denitrification

It has been proposed that in anoxic environments where nitrate is present, ANME-type archaea could link methane oxidation to denitrification (Raghoebarsing *et al.*, 2006). Indeed enrichment cultures active in both methane oxidation and nitrate reduction could be established, and ANME-type archaea were detected in the enrichments (Raghoebarsing *et al.*, 2006). Initially, it was thought that while ANME carried out AOM, the concomitant nitrate reduction was carried out by a novel type of bacteria belonging to candidate phylum NC10 (Raghoebarsing *et al.*, 2006). However, later experiments with these enrichment cultures revealed that NC10 bacteria alone could carry out both nitrate/nitrite reduction and AOM, identifying NC10 bacteria as a novel taxon capable of AOM (Ettwig *et al.*, 2010, and see below). Subsequently, the ANME-type archaea, first identified by Raghoebarsing *et al.* (2006), were demonstrated to also be capable of nitrate-dependent AOM (Haroon *et al.*, 2013). These were enriched in a laboratory-scale reactor fed with methane, nitrate and ammonium, in such conditions ANME outcompeting NC10 bacteria (see below). Insights into the biochemistry of methane oxidation in these ANME species were obtained via metagenomics, single cell genomics and metatranscriptomic analysis, in combination with stable isotope labelling of metabolites (Haroon *et al.*, 2013). In this case, methane oxidation was predicted to be through the complete reverse methanogenesis pathway, and all the genes in the pathway were highly expressed. Moreover, labelled methane was shown to be converted into labelled carbon dioxide. The same ANME species were predicted to carry out nitrate reduction. The genes for nitrate reduction (*narGH*) appeared to be of proteobacterial origin, a likely result of lateral transfer, and these were also highly expressed. Nitrite produced by the action of *narGH* was apparently utilized by the partner anaerobic ammonia-oxidizing bacteria of the genus *Kuenenia* (Planctomycetes; Haroon *et al.*, 2013). This novel partnership requiring the presence of ammonium was apparently responsible for the ANME archaea outcompeting the NC10 bacteria in nitrate-AOM.

Similar metabolic predictions were made for the ANME-type archaeon that was originally detected together with the NC10 bacterium (Raghoebarsing *et al.*, 2006), through metagenomic and metatranscriptomics sequencing (Arshad *et al.*, 2015). In this work, however, the importance of nitrite removal by a partner population, or the presence of such a partner in the bioreactor were not discussed. Interestingly, the ANME organisms involved in nitrate-AOM were found to encode and highly express an unusually high number of C-type

cytochromes, potentially suggesting their involvement in coupling AOM and denitrification (Arshad *et al.*, 2015).

Recently, genome drafts were reconstructed through, respectively, genome-centric metagenomics (Evans *et al.*, 2015) and single cell sequencing (Mwirichia *et al.*, 2016) for novel lineages of Archaea, Bathyarchaeota and MSBL1 lineage. Metabolic reconstruction from these genomes suggest roles in methane metabolism for these novel Archaea, more likely in methane oxidation than in methanogenesis, expanding the phylogenetic range of organisms capable of AOM.

New insights into methanotrophy in bacteria

'Anaerobic' methane oxidation by bacteria of the NC10 candidate phylum

Novel bacteria belonging to the Candidate phylum NC10 were highly enriched in an anaerobic reactor fed methane and nitrate (Raghoebarsing *et al.*, 2006). Subsequently it was shown that these bioreactor enrichments are better maintained if nitrite is supplied as a nitrogen source instead of nitrate (Ettwig *et al.*, 2010). Important insights into the metabolic make up of the NC10 bacteria, named *Methylomirabilis oxifera*, were gained via metagenomic sequencing of the microcosm DNA highly enriched in this organism's DNA (approximately 80% of total population). A single-scaffold sequence was generated for the genome of *M. oxifera*, revealing the presence of the complete set of genes for 'aerobic' methane oxidation, typical of other bacterial methanotrophs (Chistoserdova, 2011). Carbon assimilation was predicted to be autotrophic, using the traditional CBB cycle (Rasigraf *et al.*, 2014), as is also the case for verrucomicrobial methanotrophs (Hou *et al.*, 2008; Anvar *et al.*, 2014.) In terms of nitrate reduction, a nearly complete pathway was uncovered in the NC10 genomes through metabolic reconstruction, with the predicted product being nitrous oxide, as is typical of many denitrifying microbes (Orellana *et al.*, 2014). However, a novel pathway has been proposed instead, of dismutation of NO into oxygen and dinitrogen, to account for a potential source of oxygen (Ettwig *et al.*, 2010). While no biochemical evidence has been obtained so far for such novel metabolism, NC10 type bacteria have been detected in many environmental niches characterized by high concentrations of methane and nitrate, both terrestrial and marine (Zhu *et al.*, 2012; Deutzmann *et al.*, 2014; Padilla *et al.*, 2016), highlighting their importance in global methane cycle.

'Aerobic' methanotrophs thrive in hypoxic environments

Pure cultures of typical aerobic methanotrophs, when cultivated in laboratory, are known to thrive at high oxygen (Trotsenko and Murrell, 2008). However, this type of cultivation is very artificial, as in most natural environments methanotrophs rarely face high oxygen where methane is present. Thus, by nature, most methanotrophs must be adapted to life in oxygen-limited conditions. Obviously, methane-oxidizing performance, including doubling times seen in laboratory cultures (Soni *et al.*, 1998) cannot be expected from methanotrophs inhabiting natural environments.

From the now available genomic and metagenomic data, the metabolic makeup of the so-called 'aerobic' and the so-called 'anaerobic' bacterial methanotrophs are very similar in terms of the pathways for methane oxidation as well as for nitrate reduction (Chistoserdova,

2011, 2015). At least some aerobic methanotrophs encode genes for denitrification (Kits *et al.*, 2015a,b; Kalyuzhnaya *et al.*, 2015), and denitrification activity was experimentally demonstrated with pure cultures of typical 'aerobic' methanotrophs, induced under hypoxic conditions (Kits *et al.*, 2015a,b). In addition, many environmental studies that employed omics methods provided evidence for the activity of 'aerobic' methanotrophs under hypoxia. For example, DNA-SIP experiments with High Arctic soils in Norway identified *Methylobacter* species as the most active methane oxidizers in these environments (Graef *et al.*, 2011). Notably, in these experiments, communities from the anoxic and from the oxygen transition layers of soil displayed higher activity in methane oxidation, including oxidation of atmospheric methane, compared to the oxic layer (Graef *et al.*, 2011). *Methylobacter* sequences were also identified as most abundant methanotroph sequences in active layers of Norway arctic soils, via metagenomic and metatranscriptomic approaches (Tveit *et al.*, 2013, 2014). In these studies, *Methylobacter* sequences were once again detected in both oxic and anoxic soil layers. However, the abundance of transcripts assigned to *Methylobacter* was the highest in anoxic layers, and the most highly transcribed genes were the genes for pMMO, the enzyme that requires oxygen (Sirajuddin and Rosenzweig, 2015). A metagenomic approach was applied to test for the response of native permafrost microbial communities to thaw (Mackelprang *et al.*, 2011), revealing shifts in microbial community composition in response to a burst of methane that was trapped in the frozen soil. The responding microbes were identified through metagenomic analysis as 'aerobic' methanotrophs belonging to Alpha- and Gammaproteobacteria (Mackelprang *et al.*, 2011). Yergeau *et al.* (2010), using metagenomics, polymerase chain reaction (PCR) amplification and microarray hybridization compared communities in a permanently frozen layer dating to 5000 years ago to the communities in the active layer, finding them similar and finding the methanotroph population to be dominated by gammaproteobacterial methanotrophs. The same group carried out methane DNA-SIP experiments with the active layer sample, again finding gammaproteobacterial methanotrophs (*Methylobacter* and *Methylosarcina* types) to be active in incorporating the label (Martineau *et al.*, 2010). When metabolically active communities were investigated in stratified thermokarst shallow ponds in northern Québec, Canada, using 16S rRNA transcript profiling (Crevecoeur *et al.*, 2015), gammaproteobacterial methanotrophs, predominantly *Methylobacter*, were identified in all samples, both in the oxygenated surface waters and in hypoxic bottom waters, their relative abundance reaching up to 25% of total sequences (the highest abundance was measured in the sample with the lowest dissolved oxygen (Crevecoeur *et al.*, 2015). Blees *et al.* (2014) also presented a compelling evidence of methane oxidation proceeding via the 'aerobic' mode even when oxygen concentrations were below detection limit of common sensing devices. Interestingly, in this case, nitrate or nitrite concentrations were too low to account for the observed methane turnover (Blees *et al.*, 2014). These data further point towards a significant gap in our understanding of *in vivo* methane oxidation by 'aerobic' proteobacterial methanotrophs. A recent study that characterized an obligate microaerophilic methanotroph suggests that motility may play a key role in enabling these microbes to benefit from the optimal methane/oxygen ratios in natural environments (Danilova *et al.*, 2016).

Methane oxidation as a community function

To gain insights into the dynamics of natural microbial populations in response to oxygen availability, a microcosm approach was implemented (Oshkin *et al.*, 2015). When slurries of

Lake Washington sediment were fed methane as a single source of carbon, under either oxic or hypoxic conditions, with weekly transfers with dilutions, a very rapid loss of community complexity was observed over the course of a few weeks. In these microcosms, *Methylobacter* represented the main methane-oxidizing species. However, other, non-methanotrophic species were persisting in these communities. Of the satellite species, *Methylophilaceae* species were most relatively abundant, followed by *Flavobacteriales and Burkholderiales* (Oshkin *et al*., 2015). In these experiments, a clear separation was observed in the types of *Methylophilaceae* prevailing under 'low' oxygen versus 'high' oxygen conditions (Oshkin *et al*., 2015), hinting at specific niche adaptations for different *Methylophilaceae* ecotypes. Metagenomic sequencing was carried out for select microcosms, in order to evaluate the metabolic potential of the dominant species, through matching metagenomic sequence reads to the proxy genomes of the closest cultivated relatives. Through these analyses, methylotrophy blueprints for the dominant *Methylobacter* species were reconstructed, and these matched closely to the ones of the cultivated *Methylobacter* species (Kalyuzhnaya *et al*., 2015). The *Methylophilaceae* ecotypes were matched, respectively, to a previously cultivated *Methylophilus* ecotype and two distinct *Methylotenera* ecotypes (Beck *et al*., 2014), these ecotypes selected by different oxygen partial pressures. Some of the metabolic distinctions between different ecotypes, as revealed through metagenomics, were the variable presence of the *mxaFI* genes and of the genes for respiratory denitrification, suggesting that these may play a role in adaptation to different environmental conditions, such as oxygen levels.

The microcosm approach with Lake Washington communities was further expanded by employing a gradient of oxygen tensions, to mimic conditions in a native lake sediment (Hernandez *et al*., 2015). By analysing the composition of the communities established after a number of weeks under methane, through sequencing rRNA gene amplicons, it was demonstrated that, in support of prior observations, methane-consuming communities were dominated by two major functional types, the *Methylococcaceae* and the *Methylophilaceae*. However, different species appeared to persist under different oxygen tensions. At high initial oxygen tensions, the major players were, respectively, species of the genera *Methylosarcina* and *Methylophilus*, while at low initial oxygen tensions, the major players were *Methylobacter* and *Methylotenera*. These data suggested that oxygen availability must be at least one major factor determining specific partnerships in methane oxidation. The data also further suggested that speciation within *Methylococcaceae* and *Methylophilaceae* may be driven by niche adaptation tailored towards specific placements within the oxygen gradient. Comparative analysis of the genomes of cultivated representatives most closely related to the native populations has revealed conspicuous differences in the nitrogen metabolism potential. The *Methylosarcina* genome only encoded functions for nitrate conversion into ammonium (assimilatory denitrification; Kalyuzhnaya *et al*., 2015). In contrast, the *Methylobacter* genomes encoded, in addition, respiratory nitrate reduction functions. The *Methylobacter* genomes also contained genes predicted to encode functions essential to dinitrogen fixation, including the subunits of the Rnf complex (Kalyuzhnaya *et al*., 2015). While at this moment the potential role of dinitrogen fixation in the fitness of *Methylobacter* is not obvious, its ability to denitrify presents a mechanism by which it may be able to outcompete *Methylosarcina* during hypoxia. Likewise, while the *Methylophilus* genomes only encoded assimilatory denitrification reactions, the *Methylotenera* genomes varied in terms of their denitrification potential, from assimilatory to partial dissimilatory to complete dissimilatory (Beck *et al*., 2014; McTaggart *et al*., 2015). The denitrification capability has

been experimentally demonstrated so far in one *Methylotenera* species (Mustakhimov *et al.*, 2013).

It is tempting to speculate that nitrogen metabolism functions, and especially the denitrification capability, confer competitive advantage at low oxygen to both *Methylobacter* and *Methylotenera*. It is also possible that these organisms may exchange nitrogen species such as nitrite, nitric oxide and/or nitrous oxide. The *Methylococcaceae* definitely share carbon with the *Methylophilaceae* as well as with select non-methylotrophic heterotrophs (Kalyuzhnaya *et al.*, 2008; Beck *et al.*, 2013). One mechanism responsible for carbon release is the switch by the *Methylococcaceae* methanotrophs to a fermentative mode of metabolism during hypoxia, as has been recently demonstrated (Kalyuzhnaya *et al.*, 2013). However, microbial communities were shown to also be supported by alphaproteobacterial methanotrophs (Wei *et al.*, 2016), and in this case the biochemical mechanisms remain unknown. At this point, it remains unclear what advantage the methanotrophs may be gaining from the satellite communities.

Overall, data from the experiments described above continue to question the 'strictly' aerobic nature of the aerobic methanotrophs. While the ability to denitrify presents one mechanism for coping with hypoxia (Kits *et al.*, 2015a,b), active proteobacterial methanotrophs have also been detected in anoxic environments devoid of nitrate. In this case, it has been proposed that methane oxidation could be linked to oxygenic photosynthesis (Milucka *et al.*, 2015).

While studies of natural communities, assessed in natural settings or manipulated in laboratory, provide important insights into the function and behaviour of microbial communities active in specific biogeochemical processes, it is tempting to reconstruct simplified model communities in laboratory, in order to be able to manipulate species with defined genomes, and to enable stricter control over such communities. These are known as synthetic communities, and this approach is currently gaining momentum (Grosskopf and Soyer, 2014; Ponomarova and Patil, 2015). Remarkably, synthetic methanotrophic communities, constructed of randomly selected species, have been reported to demonstrate dramatic increase in biomass with some species combinations (Stock *et al.*, 2013). In another study, while no growth enhancement was observed, increase in methane oxidation was reported for communities versus pure methanotroph cultures (Ho *et al.*, 2014). However, no biochemical explanation or mass balance analysis were offered in these studies. In a separate study employing synthetic communities of methylotrophs, it was revealed that behaviour of particular methanotroph species differed significantly from their behaviour in situ or in manipulated natural communities (Yu *et al.*, 2016). This result highlights the fact that the behaviour and activities of complex natural communities are not easily recaptured in laboratory simulations, and, perhaps, a rational design is required to recreate 'natural' behaviour (Yu *et al.*, 2016).

New data featuring other methylotrophs

New insights into pelagic methylotrophs

In ocean waters, methanotrophs are typically present at very low abundances, constituting minor fractions of total microbial communities. Nevertheless, they are detectable via PCR amplification (Sauter *et al.*, 2012). However, methanotroph populations may experience

blooms in response to increased substrate supply, such as natural gas spills (Rivers *et al.*, 2013; Crespo-Medina *et al.*, 2014). The microbial blooms in this case were dominated by gammaproteobacteria, with a significant proportion of *Methylococcaceae*, and their abundances were positively correlated with concentrations of hydrocarbons and negatively with the dissolved oxygen concentrations (Rivers *et al.*, 2013). A significant number of transcripts, including the ones of pMMO, were assigned to *Methylobacter* and to other *Methylococcaceae* (Rivers *et al.*, 2013). As part of these methane-induced blooms, non-methanotrophic methylotroph populations have also been identified, represented by *Methylopaga* and *Methylophilaceae* species (Kessler *et al.*, 2011; Rivers *et al.*, 2013; Crespo-Medina *et al.*, 2014). *Flavobacteriaceae* DNA and transcripts were also abundant in these samples, such a response bearing striking similarities to the responses seen with lake sediment methane-utilizing communities (Oshkin *et al.*, 2015; Hernandez, 2015).

Methanotrophs are also detectable in lake water columns, through a variety of techniques, including PCR amplification of the marker genes. In a subtropical water reservoir, both alpha- and gammaproteobacterial methanotrophs were identified, along with representatives of the NC10 phylum. Once again, methanotroph sequences were generally more abundant in the oxygen-depleted zone, compared to the oxygenated zone (Kojima *et al.*, 2014). In a deep south-Alpine Lake Lugano, sequences of *Methylobacter*-type methanotrophs were detected in the anoxic but not oxic zones, coinciding with the peak in methane-oxidizing potential and suggesting that 'aerobic' methanotrophs were abundant and active in the water column below the oxic–anoxic interface (Blees *et al.*, 2014). In the well-studied lake Pavin, *Methylobacter*-type methanotrophs have also been detected in anoxic layers (Biderre-Petit *et al.*, 2011). These data strongly point towards 'aerobic' methanotrophs, especially *Methylobacter* species, being active not only in hypoxic soils and sediments, but also in oxygen-deprived water columns, further challenging the long-term dogma of 'strictly' aerobic nature of these organisms. This conclusion is further supported by analysis of spatiotemporal dynamics of bacterioplankton communities in the Gulf of Finland using massively parallel sequencing of 16S rRNA fragments, identifying methanotrophs only in hypoxic layers (Laas *et al.*, 2016).

One type of a methylotroph that is ubiquitously found in surface marine waters is the OM43 clade Betaproteobacteria of the family *Methylophilaceae* (Rappé and Giovannoni, 2003), their abundances sometimes connected to phytoplankton blooms (Morris *et al.*, 2006). However, it is not entirely clear what is their preferred substrate *in situ*. Metaproteomic analysis of natural populations has identified XoxF proteins as some of the most abundant proteins in the samples (Sowell *et al.*, 2011; Georges *et al.*, 2014). Other C1 proteins were also identifiable (Georges *et al.*, 2014), suggesting their involvement in metabolism of methanol. However, laboratory incubations of representative strain HTCC2181 demonstrated stimulation with other C1 compounds, such as methyl chloride, trimethylamine N-oxide (TMAO) or dimethylsulfoniopropyonate (DMSP; Halsey *et al.*, 2012). A synergistic mode of metabolism has been proposed to explain this phenomenon, in which carbon from methanol is directed to assimilation, while other C1 substrates are used purely for energy generation.

Representatives of the OM43 clade that form a separate phylogenetic cluster from HTCC2181 were isolated from oligotrophic waters of, respectively, Hawaiian Islands and the Red Sea (Huggett *et al.*, 2012; Jimenez-Infante *et al.*, 2015). Ecotype separation of species within the OM43 clade was further confirmed by metagenomic fragment recruitment analyses that showed trends of higher abundance in low-chlorophyll and/or high-temperature

provinces for the Hawaii-Red Sea cluster but a preference for colder, highly productive waters for the HTCC2181 cluster. Genome-genome-comparisons uncovered the potentially niche-driven ecotype differentiation, reflected in somewhat different gene inventories, including the presence of different energy conservation modules in the two OM43 subclades, potentially having an effect on their energetic yields (Jimenez-Infante *et al.*, 2015).

Few metagenomic studies addressed pelagic freshwater methylotrophs. However, recently, Salcher *et al.* (2015) enumerated pelagic *Methylophilaceae* in Lake Zurich using flow cytometry and CARD-FISH, targeting two previously detected specific groups, LD28 and PRD01a001B. High-resolution sampling across water column and over multiple years has revealed high abundance of the LD28 group (up to 4% of total cell counts), with pronounced peaks in spring and autumn–winter, correlating with peaks in primary productivity. The LD28 cells were especially abundant in the cold hypolimnion samples. The PRD01a001B group was generally less abundant, showing a single peak over four years (Salcher *et al.*, 2015). The genomes of species representing these groups of *Methylophilaceae* were sequenced by the same group. Remarkably, these were unusually small genomes, similarly to the genomes of the marine pelagic *Methylophilaceae* (Giovanoni *et al.*, 2008; Huggett *et al.*, 2012), rather than to the genomes of the *Methylophilaceae* from lake sediments (Beck *et al.*, 2014; McTaggart *et al.*, 2015). Like marine *Methylophilaceae*, the freshwater pelagic *Methylophilaceae* only encoded XoxF and not MxaFI MDH enzymes. This study concluded on the common evolutionary origin of the freshwater and marine pelagic methylotrophs, their genomes being shaped by significant genome reduction through gene loss and novel gene (such as rhodopsin genes) acquisition through horizontal transfers (Salcher *et al.*, 2015). The abundance of the pelagic *Methylophilaceae* in this study negatively correlated with nitrate concentrations, further suggesting metabolic differences between pelagic and sedimental types.

Methylotrophs in plant associations

Environments such as rice paddies generate large amounts of methane. However, much of it is consumed by the methanotrophs within the same environmental niche (Conrad, 2009). Transcript analysis, along with methane-oxidizing potential measurements, indicated that gammaproteobacterial methanotrophs are likely responsible for much of the activity (Reim *et al.*, 2012; Ma *et al.*, 2013). A metaproteogenomic approach has also been applied to rice rhizosphere environments (Knief *et al.*, 2012), uncovering high complexity of the microbial communities inhabiting these environments. While most of the proteins identified were assigned to methanogenic archaea, some of the relatively abundant proteins were of bacterial origin. Of the most relatively abundant bacterial proteins, proteins for 'aerobic' methane oxidation were identified, including both sMMO and pMMO. These were assigned to both alpha- and gammaproteobacterial methanotrophs (Knief *et al.*, 2012). In addition to the methanotroph sequences and proteins, sequences and proteins of *Methylobacterium* were detected in rice rhizosphere samples (Knief *et al.*, 2012), and these were also relatively abundant in the rhizosphere of *Arabidopsis*, along with members of *Hyphomicrobiaceae* (Lundberg *et al.*, 2012). Other methylotrophs detected in the rhizosphere through metaproteogenomics were *Methylotenera* species, as well as members of *Burkholderiales*, expressing either the MxaFI-type or the XoxF-type MDH (Knief *et al.*, 2012). These results further point towards the presence of and the activity of these groups in environments experiencing hypoxic conditions.

Metaproteogenomics have been also applied to investigate methylotroph presence and abundance in the phyllosphere of various plants (Delmotte *et al.*, 2009; Knief *et al.*, 2012). A general trend was identified, for less complex communities to inhabit the phyllosphere, compared to the rhizosphere, consistent with limited range of nutrients available in this environmental niche. However, some organisms were found to be thriving in the phyllosphere, most prominently the *Methylobacterium* species (up to 20% of total phyllosphere community; Delmotte *et al.*, 2009). The *Methylobacterium* species were shown to express proteins specific to the methylotrophic mode of life, including both MxaFI- and XoxF-MDH and other methylotrophy enzymes (Delmotte *et al.*, 2009). Methylotrophy enzymes from Gram-positive organisms (*Amycolatopsis*) have also been detected (Knief *et al.*, 2012).

Recently, a massive effort was undertaken to compare microbiomes of leafs and roots of *Arabidopsis* plants, through cultivation and genomic sequencing of respective bacterial collections, followed by assembly of defined synthetic communities (Bai *et al.*, 2015). This study uncovered some discrepancies between the natural bacterial communities inhabiting the rhizosphere and the phyllosphere and communities reproduced from assemblages of pure culture isolates, further pointing to the challenges faced by the synthetic community approach method.

Insights into C1 metabolism in non-traditional methylotrophs

Marine bacteria of the SAR11 clade are the most abundant aerobic heterotrophic bacteria in the ocean surface and some of the most successful organisms on the planet (Rappé and Giovannoni, 2003). Like pelagic *Methylophilaceae*, they are characterized by possessing extremely small genomes (Giovannoni *et al.*, 2005). Remarkably, these genomes encode a variety of functions for oxidation/demethylation of C1 compounds (Sun *et al.*, 2011). These pathways were predicted to produce energy from C1 oxidation, while no pathways were encoded for C1 assimilation. Sun *et al.* (2011) demonstrated that pure cultures of *Pelagibacter ubique,* indeed, could convert into CO_2 substrates such as methanol, formaldehyde, methylamine, glycine betaine, trimethylamine (TMA), trimethylamine oxide (TMAO), as well as dimethylsulfoniopropionate (DMSP). Elevated levels of ATP were recorded in these cultures compared to controls. Moreover, natural communities of the Sargasso Sea microbial plankton revealed similar activities (Sun *et al.*, 2011). These findings suggest that energy production from C1 compounds, rather than traditional methylotrophy, take place. A term has been coined, 'methylovory', to refer to such types of metabolism (Sun *et al.*, 2011). In a separate study, proteins implicated in C1 pathways of SAR11 were detected in coastal water samples through metaproteomics, suggesting high expression for the relevant genes (Williams *et al.*, 2012).

A similar type of metabolism was demonstrated for another representative of abundant marine heterotrophs belonging to the Marine *Roseobacter* clade. Pure cultures of *Ruegeria pomeroyi* were demonstrated to utilize TMA and TMAO as supplementary energy sources when grown on glucose. In this case as well, catabolism of TMA and TMAO resulted in the production of intracellular ATP and enhanced growth rate and growth yields, as well as enhanced cell survival during starvation (Lidbury *et al.*, 2015).

The genes encoding TMA monooxygenase (*tmm*) and TMAO demethylase (*tdm*) have been recently characterized (Chen *et al.*, 2011; Lidbury *et al.* 2014). BLAST tests with the Global Ocean Sampling dataset (Rush *et al.*, 2007) have revealed that these genes were present in approximately 20% of bacteria inhabiting surface ocean waters, most prominently

in SAR11 types (Chen *et al*., 2011; Lidbury *et al*., 2014). Moreover, through re-examining published metaproteomic and metatranscriptomic datasets, these genes were found to be highly expressed (Lidbury *et al*., 2014). Recently, it has been demonstrated that TMA monooxygenase is responsible for DMS oxidation, following a complex regulation pattern (Lidbury *et al*., 2016). All these results point to C1 metabolism by pelagic bacteria to be of significant environmental importance, potentially affecting both nutrient flow within marine surface waters and the flux of C1 compounds into the atmosphere.

A number of bacteria not formally characterized as methylotrophs encode not only modules for C1 oxidation, but also modules for C1 assimilation, which makes them potentially true methylotrophs, even if conditions for methylotrophic growth have not been established in laboratory. *R. pomeroyi* is one such example. In addition to the TMA, TMAO and DMS oxidation enzymes discussed above, along with multiple DMSP degradation pathways (Reisch *et al*., 2011), it encodes XoxF and multiple other methylotrophy enzymes and pathways (Chistoserdova, 2011).

Methylotrophy may also be overlooked in terrestrial bacteria. The recent discovery of Ln^{3+} requirement for the XoxF-MDH activity (Chistoserdova, 2016) poses questions about methylotrophy in abundant and broadly distributed guilds of bacteria such as the diverse *Burkholderiales*, the nitrogen-fixing Rhizobia, the environmentally important nitrifying bacteria, and certain animal symbionts (Chistoserdova, 2016). Confirming these guilds as methylotrophs or methylovores may significantly shift understanding of their role in C1 metabolism and expand our understanding of the role of C1 metabolism as part of the global carbon cycle.

Conclusions

While methylotrophy has been known, as a physiological phenomenon, for over one hundred years, most of the knowledge on the biochemistry, physiology and biogeochemical activities has relied on organisms available in pure cultures. The availability of the molecular tools for environmental detection has greatly expanded the means by which methylotrophs could be assessed in natural environments. Current omics technologies provide means for detecting, enumerating and measuring the activities of methylotrophs in their natural habitats, with high precision. Analysis of the recently published data presented in this review highlights the fast progress in this area and also pinpoints several emerging issues. First, it questions the 'strictly aerobic' nature of proteobacterial methanotrophs. The data presented here strongly point towards the activity of these organisms, especially the *Methylobacter* types, in microoxic and hypoxic conditions. The second and related issue is of the nature of 'anaerobic' methane oxidation by bacteria. The similarity of the schemes for carbon and nitrogen metabolism uncovered through (meta) genomics argues against such differentiation. The third emerging issue is the role of communities in environmental C1 cycling. So far, community function in methane oxidation is well supported through both SIP experiments and through analysis of stable microcosm communities. Similar patterns may exist in utilization of other C1 compounds. The final emerging issue is the role of non-traditional methylotrophs in global C1 metabolism and the role of methylotrophy pathways in non-methylotrophs. A steady stream of the omics data produced by multiple groups, with samples from a variety of environments, will collectively contribute to resolving these current challenges in understanding methylotrophy, methylotrophs and methylotroph communities.

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