Methanotrophy in Acidic Soils, Including Northern Peatlands

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Abstract

Methane oxidizing microorganisms are present and active in diverse acidic environments including peatlands, geothermal areas, and forest soils. Methanotrophic communities in acidic environments have been examined using cultivation-based physiological analyses as well as cultivation-independent molecular approaches, including omic-technologies. Most investigations have focused on moderately acidophilic, aerobic methanotrophs belonging to the phylum *Proteobacteria* that are capable of growth as low as pH 4. However, some *Verrucomicrobia* are capable of oxidizing methane aerobically at pH 1. *Alphaproteobacteria* methanotrophs generally

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dominate the methanotrophic communities in acidic oligotrophic bogs, while *Gammaproteobacteria* methanotrophs are more predominant in minerotrophic fens. The *Verrucomicrobia* methanotrophs appear to be limited to geothermal or sulfidic environments. Recent evidence has suggested that anaerobic methane oxidation may also be important in acidic peatland environments. The known diversity and metabolic potential of aerobic and anaerobic methanotrophs that are active under acidic conditions has advanced in recent years. This chapter will summarize cultivation, molecular ecology, taxonomy, and physiology studies of acidophilic methanotrophs.

Abbreviations

AOM	anaerobic oxidation of methane
FISH	fluorescence *in situ* hybridization
PLFA	phospholipid fatty acid
pMMO	particulate methane monooxygenase
SIP	stable isotope probing
sMMO	soluble methane monooxygenase

1 Introduction

Methanotrophic bacteria play an important role in the biogeochemical cycling of carbon and in mitigating the atmospheric efflux of the potent greenhouse gas methane. While methane-oxidizing communities are found in diverse habitats, some of the most important terrestrial ecosystems globally are acidic in nature (Dedysh 2009; Yu et al. 2017). Acidic terrestrial environments include peatlands, geothermally influenced areas, and many forest soils. Methane oxidation by acidophilic methanotrophs in these environments is known to occur via aerobic and anaerobic processes. Aerobic methanotrophs belong to the *Proteobacteria* and *Verrucomicrobia* phyla, while anaerobic oxidation processes have been attributed to bacteria of the candidate phylum NC10 as well as to some *Archaea*. This chapter outlines the state of our understanding of methanotrophic bacteria in acidic terrestrial environments, specifically focusing on their taxonomy and physiological mechanisms. The focus of the chapter is only on characterizing the methanotrophic microbes active in these environments, rather than on the complex ecological factors controlling net methane fluxes or the contribution of these fluxes to the global methane budget. For such biogeochemical considerations, one is referred to other recent reviews (e.g., Dean et al. 2018).

2 Methanotrophy in Peatlands

Peatlands account for ~30% of the global terrestrial soil carbon pool and represent one of the largest natural sources of atmospheric methane (Gorham 1991). The anaerobic decay of accumulated organic matter leads to the eventual formation of

methane by methanogenic *Archaea*. Atmospheric methane release is mitigated, however, by methane-oxidizing bacteria. Consuming between 10% and 90% of the methane produced (Segers 1998), aerobic methanotrophs in peatlands are found free-living in the upper, oxic-zones of peatland soil or associated with the submerged parts of mosses (Kip et al. 2010). Although this relationship with mosses has occasionally been dubbed "symbiotic" (Raghoebarsing et al. 2005), better terms are probably "moss associated methanotrophy" or "*Sphagnum*-associated methanotrophy," as the nature of the relationship, and its specificity, are not clear. Most research efforts into methane oxidation in peatlands have focused on aerobic processes; however, there is increasing evidence that anaerobic methanotrophs are also important in anoxic zones (Smemo and Yavitt 2007).

Peatlands may be either fens or ombrotrophic bogs. The latter are especially oligotrophic and acidic due to the lack of nutrients and poor buffering capacity. Measured pH values range from 3.0 to 7.0 but are typically below 5 in ombrotrophic bogs. To survive in these harsh conditions, methanotrophs must therefore be acidophilic. To date, methanotrophy in these environments has been attributed to acidophilic alphaproteobacterial and gammaproteobacterial methanotrophs, but not to the extremely acidophilic methanotroph species in the phylum *Verrucomicrobia,* which appear to be absent in these habitats (Tveit et al. 2013; Sharp et al. 2014).

A large fraction of the world's peatlands are *Sphagnum*-dominated areas in northern Russia, which account for about one-half of the world's peat (Smith et al. 2004), as well as similar ecosystems in northern Canada and Alaska. However, an estimated 11% of all peat area and 15–19% of all peat C is sequestered in tropical peatlands, particularly in Southeast Asia (Page et al. 2011). Tropical peatlands are generally also acidic in nature (e.g., Hribljan et al. 2016; Yule et al. 2016). However, research into peatland methanotrophs has to date been highly biased towards northern latitude sites, and these will necessarily be the focus of this review. Efforts to understand methane dynamics in northern wetlands are particularly important given their potential for increased methane emissions as a result of global warming. Surface warming in the Arctic is progressing at nearly twice the rate of the global average temperature increase and has already increased by 3.5 °C compared to the beginning of the twentieth century (Richter-Menge and Mathis 2017).

A variety of experimental techniques have been used to identify acidophilic, methanotrophic communities in peatlands. These include: (i) cultivation studies, (ii) cultivation-independent detection and enumeration using fluorescence *in situ* hybridization, (iii) cultivation-independent analysis of signature phospholipid fatty acids (PLFA), and (iv) cultivation-independent recovery of methanotroph-specific gene sequences and analyses of these with cloning-and-sequencing, microarrays, or high-throughput sequencing (Dumont 2014). Methanotrophs can be identified in 16S rRNA gene sequence read sets, or more specifically via sequencing of genes that encode methane monooxygenase enzymes (MMO), which catalyze the initial reaction in the methane oxidation pathway. MMO exists in both soluble (sMMO) and particulate (pMMO) forms, which are not related evolutionarily. Soluble MMO is not universal to methanotrophs, but specific phylogenetic lineages of methanotrophs possessing it can be investigated via recovery and

amplification of the *mmoX* genes encoding one subunit of sMMO. In contrast, the active-site containing subunit of pMMO encoded by *pmoA* is nearly universal among methanotrophs. The only known aerobic methanotrophs lacking pMMO are strains of the genera *Methylocella* and *Methyloferula*. Phylogenies based on *pmoA* also closely correspond to 16S-rRNA gene-based phylogenies making this an excellent tool for cultivation-independent surveying of methanotrophic communities (Knief 2015). Stable isotope probing (SIP) techniques, particularly ^{13}C-DNA-SIP, are often combined with these identification methods to assess activity of different species.

2.1 Aerobic Methane-Oxidizing *Alphaproteobacteria* in Peatlands

Early studies suggested that methanotroph communities in peat bogs are dominated by the genera *Methylocella*, *Methylocapsa*, and *Methylocystis*, which all belong to the class *Alphaproteobacteria* (Dedysh et al. 2001; Chen et al. 2008a). Cultivation-independent molecular recovery of *pmoA* and *mmoX* genes from various peatlands has repeatedly demonstrated an abundance of *Methylocystis* (McDonald et al. 1997; Morris et al. 2002; Jaatinen et al. 2005; Chen et al. 2008a, b; Siljanen et al. 2011; Putkinen et al. 2012). Chen et al. (2008b) used $^{13}CH_4$ DNA-SIP to demonstrate that *Methylocystis* spp. were the most active methanotrophs in six of eight studied peat bogs ranging from pH 4.2 to 4.9. Recovery of *pmoA* mRNA transcripts of *Methylocystis* from Moor House Peat in the UK also indicated that these bacteria were active (Chen et al. 2008a). Chen et al. (2008a) also detected a predominant group ("MHP" clade) of *pmoA* sequences somewhat related to *Methylocapsa* in *Calluna*-covered moorlands (pH 4.6) of the UK.

In the *Sphagnum-Carex* Bakchar bog (pH 3.6–4.5) of western Siberia, and in a *Sphagnum* peat bog lake (pH 4.2) in Germany, methanotrophs were enumerated using a set of 16S rRNA-targeted FISH probes independently targeting *Methylocella palustris*, *Methylocapsa acidiphila*, *Methylosinus* spp., *Methylocystis* spp., and methanotrophic *Gammaproteobacteria*. The *Alphaproteobacteria* were abundant: *Methylocella palustris* ($>10^6$ cells g^{-1} of wet peat in Bakchar), *Methylocystis* spp. ($>10^6$ cells g^{-1} in both sites), and *Methylocapsa acidiphila* ($>10^5$ cells g^{-1} in both sites), while the *Gammaproteobacteria* accounted for $<1\%$ of the methanotroph populations (Dedysh et al. 2001, 2003).

This trend is supported by cultivation efforts. Most cultivated and taxonomically described methanotrophs obtained from peatlands (Table 1) belong to the class *Alphaproteobacteria* and either the family *Methylocystaceae* (*Methylocystis*) or *Beijerinckiaceae* (*Methylocella*, *Methyloferula*, *Methylocapsa)*. Validated species of *Methylocella* isolated from peat include *Methylocella palustris* (type strain, K) and *Methylocella tundrae* (type strain, T4) (Dedysh et al. 2000, 2004). These strains lack a pMMO and have only a sMMO to catalyze methane oxidation. They can be further differentiated from other proteobacterial methanotrophs by their lack of intracellular membrane stacks. Rather, *Methylocella* spp. have vesicular membrane invaginations that are connected to the cytoplasmic membrane. *Methylocella*

Table 1 Characteristics of isolated, acidophilic methanotrophs from peatland, geothermal, and forest soil environments

Strains[a]	pH range (optimum)	Type of MMO	Intracellular membrane	Carbon fixation pathway	Metabolic capability
Peatlands					
Alphaproteobacteria					
Methylocapsa acidiphila B2	4.2–7.2 (5.0–5.5)	pMMO	Type III: a single membrane stack along one side of the envelope	Serine	Obligate methanotroph
Methylocapsa palsarum NE2	4.1–8.0 (5.2–6.5)	pMMO	Type III: a single membrane stack along one side of the envelope	Serine	Obligate methanotroph
Methylocella palustris K	4.5–7.0 (5.0–5.5)	sMMO	Vesicular membrane invaginations connected to the cytoplasmic membrane	Serine	Facultative methanotroph
Methylocella tundrae T4	4.2–7.5 (5.5–6.0)	sMMO	Vesicular membrane invaginations connected to the cytoplasmic membrane	Serine	Facultative methanotroph
Methylocystis bryophila H2s	4.2–7.6 (6.0–6.5)	pMMO; sMMO	Type II: paired membrane stacks along the cell periphery, parallel to the envelope	Serine	Limited facultative methanotroph[b]
Methylocystis heyeri H2	4.4–7.5 (5.8–6.2)	pMMO; sMMO	Type II: paired membrane stacks along the cell periphery, parallel to the envelope	Serine	Limited facultative methanotroph[b]
Methyloferula stellata AR4	3.5–7.2 (4.8–5.2)	sMMO	Vesicular membrane invaginations connected to the cytoplasmic membrane	Serine; RuBP	Obligate methanotroph
Gammaproteobacteria					
Candidatus Methylospira mobilis	4.2–6.5 (6.0–6.5)	pMMO	Type I: a bundle of vesicular disks	RuMP	Obligate methanotroph
Methylobacter tundripaludum SV96	5.5–7.9	pMMO	Type I: a bundle of vesicular disks	RuMP	Obligate methanotroph
Methylomonas paludis MG30	3.8–7.3 (5.8–6.4)	pMMO	Type I: a bundle of vesicular disks	RuMP	Obligate methanotroph

(*continued*)

Table 1 (continued)

Strains[a]	pH range (optimum)	Type of MMO	Intracellular membrane	Carbon fixation pathway	Metabolic capability
Geothermal environments					
Verrucomicrobia					
"Methylacidimicrobium cyclopophantes" 3B	0.6–3.0 (1.5–3.0)	pMMO	ND	CBB	Obligate methanotroph
"Methylacidimicrobium fagopyrum" 3C	0.6–6.0 (1.5–3.0)	pMMO	Membrane stacks orthogonal to the cytoplasmic membrane	CBB	Obligate methanotroph
"Methylacidimicrobium" sp. LP2A	1.0–5.2 (3.1)	pMMO	ND	CBB	Obligate methanotroph
"Methylacidimicrobium tartarophylax" 4AC	0.5–6.0 (1.0–3.0)	pMMO	ND	CBB	Obligate methanotroph
"Methylacidiphilum fumariolicum" SolV	0.8–5.8	pMMO	Carboxysome-like structures	CBB	Methanotroph; $H_2 + CO_2$
"Methylacidiphilum infernorum" V4	1.0–6.0 (2.0–2.5)	pMMO	Carboxysome-like structures	CBB	Obligate methanotroph
"Methylacidiphilum kamchatkense" Kam1	2.0–5.0 (3.5)	pMMO	Carboxysome-like structures	CBB	Obligate methanotroph
Forest soils					
Alphaproteobacteria					
Methylocapsa aurea KYG	5.2–7.2 (6.0–6.2)	pMMO	Type III: a single membrane stack along one side of the envelope	Serine	Limited facultative methanotroph[b]
Methylocella silvestris BL2	4.5–7.0 (5.5)	sMMO	Vesicular membrane invaginations connected to the cytoplasmic membrane	Serine	Facultative methanotroph
Gammaproteobacteria					
Methylovulum miyakonense HT12	6.0–7.5	pMMO; sMMO	Type I: a bundle of vesicular disks	RuMP	Obligate methanotroph

Abbreviations: *CBB* Calvin–Benson–Basham cycle, *ND* not detected or not reported, *RuBP* ribulose bisphosphate pathway, *RuMP* ribulose monophosphate pathway
[a]Strains have been limited to type strains only
[b]Growth on acetate, in addition to methane/methanol, has been reported

palustris and *Methylocella tundrae* are capable of growth at pH 4.5–7.0 (optimum 5.0–5.5) and pH 4.2–7.5 (optimum 5.5–6.0), respectively. An additional unique characteristic of *Methylocella* spp. is their ability to grow on various compounds containing carbon-carbon bonds in addition to methane. This metabolic capability identified some *Methylocella* spp. as the first facultative methanotrophs and the most

catabolically versatile of all known methanotrophs (Dedysh et al. 2005; Dedysh and Dunfield 2018).

Methyloferula stellata (type strain, AR4) is another species known to encode only sMMO and not pMMO (Vorobev et al. 2011). Isolated from *Sphagnum* bogs in Russia, this methanotroph is moderately acidophilic with an optimum pH for growth between 4.8 and 5.2 (Table 1). Unlike *Methylocella* spp., *Methyloferula stellata* is an obligate methanotroph. It is also reported to fix carbon via the ribulose-bisphosphate pathway in addition to the serine pathway common to most alphaproteobacterial methanotrophs.

Methylocella and *Methyloferula* are among the least well studied of all methanotrophs using cultivation-independent studies, because they lack a pMMO enzyme and therefore cannot be detected via *pmoA* recovery, the method of choice for the majority of microbial ecology studies. Consequently, their importance relative to other species is poorly elucidated. Even detecting these genera via 16S rRNA genes sequencing is problematic, since unlike the *Gammaproteobacteria* methanotrophs or the other *Alphaproteobacteria* methanotrophs in the family *Methylocystaceae*, the methanotrophs within the family *Beijerinckiaceae* are closely related (up to 97% 16S rRNA gene sequence identity) to non-methanotrophs, and it is often difficult to discern if a sequence detected is or is not from a methanotroph. However, *Methylocella*-like *mmoX* sequences have been found in peat ecosystems (Chen et al. 2008a; Gupta et al. 2012) and the genus has been detected via 16S rRNA targeted FISH in some studies (Dedysh et al. 2001, 2003).

Methylocapsa acidiphila B2^T and *Methylocapsa palsarum* NE2^T are moderately acidophilic bacteria isolated from *Sphagnum*-rich environments in Siberia and Norway, respectively (Dedysh et al. 2002, 2015). These strains of *Methylocapsa* are obligate methanotrophs, encode a pMMO enzyme, and have an intracellular membrane system (Table 1). Both strains can grow over a broad pH range, with pH 4.2–7.2 supporting growth for *Methylocapsa acidiphila* and 4.1–8.0 for *Methylocapsa palsarum*.

Most described *Methylocystis* cultures are neutrophilic, but two species, *Methylocystis heyeri* (type strain, H2^T) and *Methylocystis bryophila* (type strain, H2s^T), are moderately acidophilic (Dedysh et al. 2007; Belova et al. 2013). The strains contain both sMMO and pMMO (Table 1), with the latter strain possessing two distinct *pmo* operons. Genomic analyses of *Methylocystis bryophila* S285 has further identified that in addition to two canonical pMMO-encoding operons *pmoCAB*, this specific strain also contains a third, highly divergent *pxmABC*-gene cluster (Fig. 1) (Han et al. 2018). The *pxmABC*-cluster has been identified primarily in gammaproteobacterial methanotrophs and a limited number of alphaproteobacterial methanotrophs, but its functional role has not yet been identified (Tavormina et al. 2011; Han et al. 2018). Some strains of *Methylocystis* have also been shown to have slow but sustained growth on acetate, identifying them as [limited] facultative methanotrophs (Belova et al. 2011).

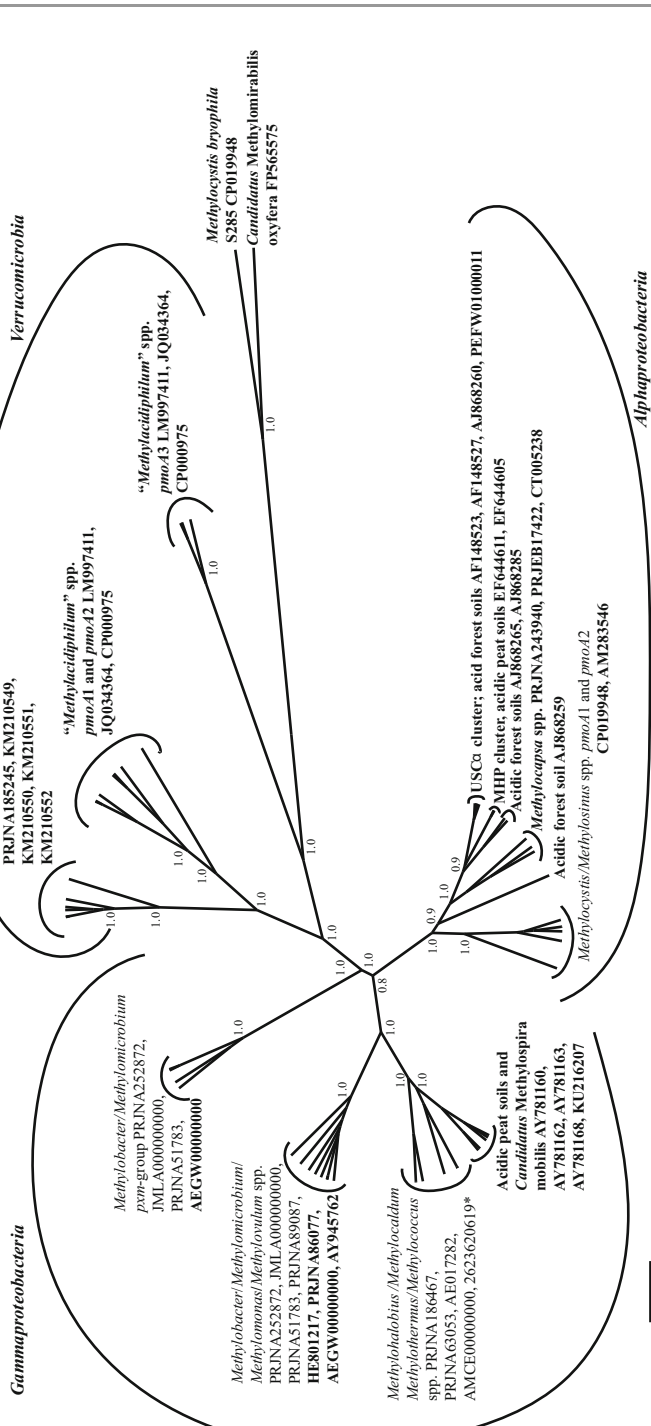

Fig. 1 Phylogenetic analyses comparing acidophilic methanotrophs to select reference strains based on partial *pmoA* gene sequences. Sequences taken from acidophilic isolates or acidic environments are in ***bold***. Sequences used for tree construction were taken from genome sequencing projects, were determined from cultured isolates or via cultivation-independent studies from acidic environments as reported by Holmes et al. (1999), Chen et al. (2008a), Knief et al. (2005, 2006), and Jaatinen et al. (2005), and are identified by their associated GenBank gene/genome accession numbers. The *asterisk* (*) symbol denotes

2.2 Aerobic Methane-Oxidizing *Gammaproteobacteria* in Peatlands

Detection and quantification of a "signature" PLFA of gammaproteobacterial methanotrophs, C16:1ω8c, has been used as evidence that these bacteria are abundant in peatlands (Krumholz et al. 1995; Sundh et al. 1995). However, the subsequent detection of this PLFA in the acidophilic alphaproteobacterial methanotroph *Methylocystis heyeri* means that it can no longer be used as an indicator of any particular group (Dedysh et al. 2007).

However, more recent sequencing studies have provided new insights into the diversity of peat-associated methanotrophic communities and indicated an important role for *Gammaproteobacteria*. Using a *pmoA*-based microarray to investigate a *Sphagnum* peat bog (pH 3.8–4.3) in the Netherlands, Kip et al. (2011a) showed a high abundance of both *Alphaproteobacteria* (*Methylocystis*, *Methylosinus*) and *Gammaproteobacteria* (*Methylobacter*, *Methylomonas*, *Methylomicrobium*) methanotrophs. Additional support for the prevalence of both lineages was provided by pyrosequencing *pmoA* amplicons from the same environment (Kip et al. 2011a). Gammaproteobacterial reads comprised 58% of the entire dataset, while 40% of the reads could be mapped to *Alphaproteobacteria*. In an independent study that combined metagenomics with sequencing analyses of *pmoA* cDNA amplicons, Esson et al. (2016) also discovered that methanotrophic communities in an acidic peat bog (pH 3.5–4.0) were co-dominated by *Methylocystis* and Methylomonas. *Sphagnum* mosses from three alpine bogs in Austria were found to support a combination of *Methylomonas* and *Methylocystis* as moss-associated methanotrophs (Bragina et al. 2013).

Other studies have even detected a marked predominance of *Gammaproteobacteria* methanotrophs in some sites. Species related to *Methylobacter tundripaludum* and *Methylobacter psychrophilus* were detected as the major methanotrophs in two mildly acidic Arctic fens at pH 5–6 via pyrosequencing of 16S rRNA genes and analysis of mRNA transcripts (Tveit et al. 2013, 2014). While multiple factors can contribute to the structural dynamics of methanotrophic communities in different sites, these studies provide evidence that both gammaproteobacterial and alphaproteobacterial methanotrophs can be important.

Compared to the *Alphaproteobacteria*, fewer isolates of acidophilic methanotrophs from the *Gammaproteobacteria* are available, and most show only a mildly acidophilic phenotype. Isolated from mossy Arctic soil, *Methylobacter psychrophilus* Z-0021^T was one of the first cultivated psychrophilic methanotrophs (Omelchenko et al. 1996; Tourova et al. 1999). *Methylobacter*

Fig. 1 (continued) sequences taken from the Integrated Microbial Genomes database with the associated IMG Genome ID number. Sequences were aligned using Clustal Omega (Sievers et al. 2011) and the tree constructed via Bayesian analyses using the BEAST2 software package (Bouckaert et al. 2014) with the general-time-reversible substitution model for ten million generations sampling every 1000th tree. Posterior probability values of major nodes are shown

psychrophilus-like strains have been detected in mildly acidic fens (Tveit et al. 2013, 2014), while other environmental studies have suggested a greater predominance of these strains in pH-neutral Arctic environments (Berestovskaya et al. 2002; Martineau et al. 2010). The latter observations coincide with the growth characteristics reported for the type strain, which include a pH range of 5.9–7.6 with an optimum of 6.7 (Trotsenko and Khmelenina 2005). Unfortunately, the original culture of Z-0021^T has been lost (Graef et al. 2011). A closely related strain, *Methylobacter tundripaludum* SV96^T, was isolated from a mildly acidic, wetland soil from Svalbard (Wartiainen et al. 2006) and has been reported to grow well from pH 5.5 to 7.9. Using meta-transcriptomics, Tveit et al. (2015) identified a high abundance of pMMO and SSU rRNA transcripts that most closely matched to *Methylobacter tundripaludum* sequences in Arctic peat soils and suggested these strains were the dominant active methanotrophs. Graef et al. (2011) also identified that strains of *Methylobacter*, namely, *Methylobacter tundripaludum*, dominated the methanotrophic community found in a high Arctic wetland. *Methylobacter tundripaludum* and related strains seem to be well adapted to methanotrophy in cold-environments, which can include neutral and mildly acidic habitats. A draft genome of strain SV96^T was also released in 2011 (Svenning et al. 2011). A notable outcome of the resulting analyses was the identification of a *pxm*-version of the MMO-encoding operon in addition to the canonical *pmoCAB* operon encoded within the genome (Fig. 1).

Kip et al. (2011b) reported the growth of cultures of *Methylomonas* and *Methylovulum*-like methanotrophs from peat that were capable of growth as low as pH 3.5–4.1; however, these have never been taxonomically characterized and validated. Danilova et al. (2013) recently described the first *Methylomonas* species from an acidic (pH 3.9) *Sphaghum* peat bog. *Methylomonas paludis* MG30^T has an optimum pH between 5.8 and 6.4, possesses a pMMO, and is an obligate methanotroph capable of growing only on methane and methanol (Table 1). Recently a spiral-shaped methanotroph related to the genera *Methylocaldum* and *Methylococcus* (sometimes called the type Ib methanotrophs) was enriched from *Sphagnum* peat and dubbed *"Candidatus* Methylospira mobilis" (Danilova et al. 2016b). Although its optimum pH is only 6.0–6.5, it is capable of growth down to pH 4.2. This species appears to be widespread in acidic peat bogs based on *pmoA* analysis (Danilova et al. 2016a, b).

2.3 Are There Patterns to Methanotroph Community Structure Across Peatlands?

Clearly, many different methanotroph species, both *Gammaproteobacteria* and *Alphaproteobacteria*, have been identified as predominantly abundant, or predominantly active, in different peatlands. Extrapolating general patterns is complicated not only because every site is unique, but also because nearly every study is also unique and employs a different set of methods. Communities may be identified based on 16S rRNA, *pmoA,* and/or *mmoX* genes, and the actual DNA sequence

variant identifications may be made through denaturing gradient gel electrophoresis, FISH, cloning and sequencing, microarray analysis, or high-throughput sequencing. Some studies include a functional analysis of active species using SIP or transcriptome analyses. Add to this a host of other differences in sampling time and intensity, sample handling, DNA extraction methods, PCR protocols, etc., and comparison becomes somewhat problematic.

Nevertheless, some general trends do emerge. Comparatively nutrient-rich, only mildly acidic (pH >5) fens often show a strong predominance of *Gammaproteobacteria* methanotrophs (Jaatinen et al. 2005; Tveit et al. 2013, 2014; Christiansen et al. 2014), whereas oligotrophic, highly acidic (pH <5) ombrotrophic bogs are more likely to show a strong predominance of *Alphaproteobacteria* methanotrophs (Dedysh et al. 2001; Chen et al. 2008b; Gupta et al. 2012; Kravchenko et al. 2015). Many sites are intermediate and contain both groups.

Studies that simultaneously compare different sites with the same methods are particularly useful when drawing this conclusion, and the pattern of increased *Alphaproteobacteria* methanotroph dominance with increasing acidity and nutrient limitation is surprisingly consistent in these studies. Gupta et al. (2012) compared a nutrient-rich sedge fen to a nutrient-poor *Sphagnum* bog using a combination of ^{13}C-DNA SIP and fingerprinting via *pmoA* and *mmoX*. The bog was predominated by *Alphaproteobacteria* methanotrophs (*Methylocystis*, *Methylosinus*, and *Methylocella*), whereas both *Alphaproteobacteria* and *Gammaproteobacteria* methanotrophs were present and active in the fen. Comparison of a pristine bog (pH 3.8) to adjacent drained sites (pH 4.2–4.8), via *pmoA* gene sequencing, indicated that *Methylocystis* were dominant in the pristine site, while *Methylobacter* became more abundant in the drained site (Kravchenko et al. 2015). In a comparison of a pristine ombrotrophic bog (pH 4.4–4.5) with a minerotrophic fen (pH 4.9–5.2), *Gammaproteobacteria* methanotrophs were detected as the only methanotrophs in the nutrient-rich fen, whereas in the ombrotrophic bog *Methylocystis* were detected along with the *Gammaproteobacteria* (Jaatinen et al. 2005). A comparison of hillock and hollow features of a Siberian bog was made with pyrosequencing of 16S rRNA genes (Grodnitskaya et al. 2018). The hillocks were notably more acidic and nutrient-poor and were dominated by *Alphaproteobacteria* methanotrophs (*Methylosinus* and *Methylocapsa*). In the hollows, both *Gammaproteobacteria* and *Alphaproteobacteria* methanotrophs were detected (Grodnitskaya et al. 2018).

In a more extensive survey, Putkinen et al. (2014) combined *pmoA* microarray studies with *pmoA* and 16S rRNA gene SIP analysis of 17 peatlands that represented a chronosequence of successional stages during the development of a wet meadow into a minerotrophic fen and finally an ombrotrophic bog. They observed a pattern whereby the *Alphaproteobacteria* methanotrophs (*Methylocystis, Methylosinus, Methylocella*, and *Methylocapsa*), plus the *Gammaproteobacteria* methanotroph *"Ca.* Methylospira mobilis," were most active in the late-stage ombrotrophic bogs that had the lowest nutrient availability and pH. Other *Gammaproteobacteria* (esp. *Methylobacter* and *Methylomonas*) methanotrophs were active at all sites, but particularly in the earlier fen stages.

Putkinen et al. (2014) interpreted their results as a consequence of their differing ecological life strategies of the different methanotrophic groups. The trend was consistent with early theories of the niche differentiation of *Gammaproteobacteria* versus *Alphaproteobacteria* methanotrophs, which proposed that *Gammaproteobacteria* methanotrophs thrive in favorable habitats selecting for a high growth rate, or are r-selected. Conversely, the *Alphaproteobacteria* are better suited to more stressful, less optimal habitats, and represent K-selected, or more appropriately L-selected or stress-tolerant bacteria (Hanson and Hanson 1996; Ho et al. 2013; Knief 2015). Ho et al. (2013) have summarized methanotroph strategies using a similar three-member system of Competitor, Stress tolerator, or Ruderal. Both of these classification systems are consistent with some known physiological properties that distinguish the two methanotroph groups. For the *Alphaproteobacteria* methanotrophs, these include: the ability to survive under lower methane concentrations (Knief and Dunfield 2005), a higher prevalence of nitrogenase and the common formation of cysts (Hanson and Hanson 1996), and an ability to grow in extremely low nutrient, oligotrophic media, which is a defining characteristic of all *Beijerinckiaceae* methanotrophs, including *Methylocella* and *Methylocapsa* (Dedysh et al. 2000, 2004, 2015; Vorobev et al. 2011). Conversely the *Gammaproteobacteria* methanotrophs have a more efficient C fixation pathway and generally higher growth rates (Hanson and Hanson 1996) and tend to respond more positively to nitrogen fertilization (Ho et al. 2013). The generalization that all *Gammaproteobacteria* methanotrophs are r-selected and all *Alphaproteobacteria* methanotrophs are L-selected is certainly an oversimplification and should not be applied to all members of either group. Nevertheless, as a general rule governing the distribution of species in ombrotrophic bogs versus minerotrophic fens, there appears to be some truth in it.

2.4 Anaerobic Methane Oxidation in Peatlands

There is increasing evidence of the important role that anaerobic methane oxidation (AOM) plays in peatlands. More than a decade ago, Smemo and Yavitt (2007) found that AOM can occur simultaneously with methanogenesis in a variety of peatlands and can consume large amounts of the methane produced. More recently, Gupta et al. (2013) confirmed that AOM can be widespread across diverse latitudes and peatland type. In this study, AOM was observed in both fen and bog wetlands at pH values ranging from 3.6 to 5.9. Despite increasing amounts of flux-based evidence, mechanistic insights into this process have just begun to appear.

AOM is now known to be coupled to a variety of possible electron acceptors other than molecular oxygen. In marine environments, anaerobic methanotrophic (ANME) *Archaea* can form synergistic consortia with sulfate-reducing bacteria allowing for methane to be oxidized using sulfate as the terminal electron acceptor. Other terminal electron acceptors like iron, manganese, nitrite, and nitrate have been reported to be used in methane oxidation and should be more thermodynamically favorable than

Table 2 Reactions and associated free energies under standard conditions for possible terminal electron acceptors relevant to methane oxidation

Reaction	ΔG^O (kJ mol^{-1} CH_4)[a]
$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$	−842.3
$CH_4 + SO_4^{2-} + 2H^+ \rightarrow CO_2 + H_2S + 2H_2O$	−92.8
$CH_4 + 4NO_3^- \rightarrow CO_2 + 4NO_2^- + 2H_2O$	−503.4
$3CH_4 + 8NO_2^- + 8H^+ \rightarrow 3CO_2 + 4N_2 + 10H_2O$	−929.0
$CH_4 + 8Fe^{3+} + 2H_2O \rightarrow CO_2 + 8Fe^{2+} + 8H^+$	−454.6
$5CH_4 + 8MnO_4^- + 24H^+ \rightarrow 5CO_2 + 8Mn^{2+} + 22H_2O$	−1028.1

[a]Values as reported in Caldwell et al. (2008) or Welte et al. (2016)

sulfate-coupled processes (Table 2) (Beal et al. 2009; Smemo and Yavitt 2011; Zhu et al. 2012; Ettwig et al. 2016). Assessment of AOM processes in peatlands using these electron acceptors is still somewhat limited, however. Peatlands are often considered metal- and nutrient-poor environments that, with a few exceptions, also generally have low concentrations of sulfate and nitrate, making the use of these alternative acceptors improbable (Smemo and Yavitt 2011). An alternative hypothesis is that AOM-catalyzing microbes use humic acids to shuttle electrons to metal-reducing organisms or to deeper anoxic peat (Smemo and Yavitt 2011). Humic substances are known to transfer electrons under anoxic conditions, and this process would be plausible given the organic-rich nature of peatlands, but the involvement of this mechanism in AOM-processes has not yet been explored.

Nitrite-dependent AOM has been reported in a *Sphagnum*-dominated peatland fed by nitrate-enriched groundwater (Zhu et al. 2012). Analyses of porewater samples found that nitrate and methane had depth counter-gradients with nitrate decreasing at lower depths, while methane concentrations increased. A transition zone where both compounds were depleted was identified. This transition region correlated with an increased abundance of the bacterium *"Candidatus* Methylomirabilis oxyfera" – an organism proposed to catalyze nitrate/nitrite-dependent methane oxidation. Zhu et al. (2012) further showed that enrichment cultures could achieve nitrite-dependent methane oxidation across a pH range from 5.9 to 7.5 identifying the acidophilic potential of this process. Additional support for the involvement of "*Candidatus* Methylomirabilis oxyfera" in nitrite-dependent AOM was later provided in a study linking stable-isotope analyses to bacterium-specific 16S rRNA gene-qPCR primers in both acidic and neutral wetlands (Hu et al. 2014).

In Candidatus "Methylomirabilis oxyfera"-like cultures, methane oxidation is proposed to occur via canonical aerobic methane oxidation pathways. This pathway is proposed despite the observation that methane is oxidized under anaerobic conditions and evidence that methane and nitrite conversion rates decrease at elevated O_2 concentration (Luesken et al. 2012). Instead of consuming O_2 from the surrounding environment, the molecular oxygen needed for methane oxidation may be produced by the bacterium through the dismutation of nitric oxide into dinitrogen gas and O_2 (Zhu et al. 2012).

Nitrate-driven AOM has also been reported for a specific ANME-archaeal lineage (ANME-2d) described as "*Candidatus* Methanoperedenaceae" (Haroon et al. 2013). Unlike Candidatus Methylomirabilis oxyfera-like organisms, this lineage is proposed to catalyze methane-oxidation via reverse methanogenesis with the initial reaction catalyzed by methyl-CoM reductase. Additionally, this lineage is not thought to be capable of AOM independently and is instead reliant on the syntrophic feeding of nitrite to an ammonium-oxidizing bacterium (Haroon et al. 2013). It is important to note, however, that while reverse methanogenesis in peatlands is considered possible (Blazewicz et al. 2012), the experiments used to describe the ANME-2d mechanisms of AOM were done in neutrophilic bioreactors.

3 Geothermally Influenced Environments

Geothermal environments are characterized by high temperatures and are frequently also acidic due to the oxidation of sulfur compounds. While methane concentrations in geothermal gas are typically <1%, some geothermal systems can have molar fractions of methane between 1% and 11% (v/v gas) with anomalies up to 27% (Giggenbach 1995; Etiope and Klusman 2002). The first evidence of extremely acidic methane oxidation was observed in geothermal soils in the Solfatara volcano region near Naples, Italy, a site characterized by high temperature (50–95 °C) and pH as low as 1.0 (Castaldi and Tedesco 2005). Later, atmospheric methane release in steaming geothermal surface soils in New Zealand was found to be mitigated by methanotrophic bacteria (Dunfield et al. 2007). In 2007, cultured isolates were simultaneously reported from three acidic geothermal sites, including an acidic hot spring in Kamchatka, Russia (isolate Kam1), the Solfatara volcano region (isolate SolV) and steaming soil at Tikitere, New Zealand (isolate V4) (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008). These isolates had a pH optimum of 2.0–3.5 with a lower limit of 0.8 (Table 1) and could grow at temperatures up to 65 °C. Based on 16S rRNA gene analyses, all three isolates formed a single genus-level cluster within the *Verrucomicrobia* phylum, identifying them as the first methanotrophs outside of the *Proteobacteria*. This cluster is currently described as "*Methylacidiphilum*," but this taxonomic designation remains to be validated.

A second, not-yet validated genus of methanotrophic *Verrucomicrobia*, called "*Methylacidimicrobium,*" has more recently been described (Sharp et al. 2014; van Teesling et al. 2014). This genus is comprised of mesophilic isolates from the Solfatara crater (Naples, Italy) and a geothermal soil in New Zealand. The demonstration that both mesophilic and thermophilic strains of methanotrophic *Verrucomicrobia* exist suggests this phylotype may be more widespread than presently known.

Putative methanotrophic *Verrucomicrobia* were found in several environments over a wide temperature range (22.5–81.6 °C) in New Zealand (Sharp et al. 2014). However, Sharp et al. (2014) detected them only in natural acidic environments (pH <5) that were geothermally influenced, and not in acidic bogs. Evidence for

these bacteria has also been identified by 16S-rDNA sequence analyses in sulfide-corroded sewage pipes (Pagaling et al. 2014), as well as at multiple depths in a nitrogen-fertilized paddy soil (Vaksmaa et al. 2017). In the latter case, however, the sequences shared only 85% nucleotide sequence identity with cultured representatives of the "*Methylacidiphilum*" group, and the involvement of these bacteria in methane oxidation should be viewed very cautiously pending further investigation.

With the exception of a few facultative methanotrophs, methane-oxidizing bacteria have limited metabolic versatility (Dunfield and Dedysh 2014). Recent investigations involving "*Methylacidiphilum*" strains are now challenging our understanding of these niche limitations in methanotrophic *Verrucomicrobia*, however. Isolate SolV has been reported to grow on hydrogen and carbon dioxide in the absence of methane (Mohammadi et al. 2017), which is mediated by two distinct uptake hydrogenases. The two hydrogenases differ in their sensitivity towards oxygen, and RNA-seq analyses have shown differences in expression levels of the encoding genes as a function of oxygen concentration. Carere et al. (2017) have further shown that "*Methylacidiphilum*" sp. RTK17.1 is capable of mixotrophic growth, whereby methane and H_2 can be consumed simultaneously to support respiration and carbon fixation. Given these new insights into the metabolic versatility of some of these strains, the number of possible ecological niches occupied by this group of methanotrophs may be larger than currently recognized.

Like their aerobic, proteobacterial counterparts, the first reaction in methane oxidation by methanotrophic *Verrucomicrobia* is catalyzed by MMO enzyme complexes. Genomic analyses of cultivated "*Methylacidimicrobium*" and "*Methylacidiphilum*" species have only identified pMMO encoding genes and no sMMO-encoding homologues have been identified (Pol et al. 2007; Hou et al. 2008; Anvar et al. 2014; Sharp et al. 2014; van Teesling et al. 2014; Erikstad and Birkeland 2015). Strains in the genus "*Methylacidiphilum"* contain three complete *pmoCAB* operons, which are known to vary from each other by up to 50% in amino acid sequence (Fig. 1). In silico analyses have provided evidence that one of these operons, *pmoCAB*3, has been obtained through lateral gene transfer rather than paralogous replication (Sharp et al. 2013). In contrast, "*Methylacidimicrobium*" strains have only a single *pmoCAB* operon, except strain LP2A, which has two, near-identical operons (Fig. 1). "*Methylacidiphilum kamchatkense"* Kam 1 and "*Methylacidimicrobium*" sp. LP2A genomes also encode orphan *pmoC* genes, but the role of these orphan genes, if any, is not yet clear.

"*Methylacidimicrobium"* strain 3C is the only strain of either genus known to contain intracytoplasmic membrane stacks, which is a trait found in pMMO-encoding proteobacterial methanotrophs (Table 1). Rather, "*Methylacidiphilum"* species are known to contain carboxysome-like compartments. These structures may serve to anchor pMMO enzymes, but further investigation to confirm this hypothesis is required. Alternatively, they may simply play a role as glycogen storage vesicles (Khadem et al. 2012). *Verrucomicrobia* methanotrophs fix carbon dioxide via the Calvin-Benson-Basham cycle, rather than assimilate it via the ribulose-monophosphate or serine pathways found in gammaproteobacterial and alphaproteobacterial methanotrophs, respectively (Table 1).

4 Other Soils

Many terrestrial soils and sediments are mildly acidic. A thorough examination of the methanotrophs in all such sites would be well beyond the scope of this brief review. Nevertheless, some mention should be made of the putative USCα group of proposed atmospheric methane oxidizers present in primarily acidic forest soils.

Estimates of global methane uptake by oxic, well-aerated upland soil vary widely, but current estimates fall within the range of 9–47 Tg C^{-1} $year^{-1}$ (Ciais et al. 2013). Of these, forest soils are estimated to consume ~2.5 times more methane globally than grassland soils (Yu et al. 2017). The source of methane for these populations is the overlying atmosphere, where globally averaged mixing ratios are ~1.83 ppmv (Yu et al. 2017). At these low levels, and with the realization that most forest soils are moderately acidic (pH 4–6), it stands to reason that methanotrophic populations in forest soils are both acidophilic and oligotrophic. Kinetic responses for methane uptake in forest soils follow a typical Michaelis-Menten curve in response to methane concentration, but the apparent affinity is orders of magnitude higher in soils (10–100 nM) than in methanotrophic cultures (1–10 μM). This suggests that the methanotrophs in these soils possess a high-affinity version of MMO allowing them to survive on trace levels of atmospheric methane (Knief et al. 2006)

The cultivation of these "high-affinity" methane oxidizers found in upland soils has not yet been successful. Holmes et al. (1999) were the first to describe atmospheric methane-oxidizing bacterial communities in three acidic forest soils (pH 3.4–4.9). Incubation of the soils under an atmosphere of $^{14}CH_4$ at low concentrations (<50 ppmv) allowed for the ^{14}C-labelled phospholipid fatty acid (PLFA) profiles of the "high-affinity" methanotrophic populations to be determined. The recovered PLFAs were most similar to acidophilic alphaproteobacterial methanotrophs, namely, *Methylocapsa* spp. Based on sequence phylogeny, Holmes et al. (1999) also discovered that *pmoA* sequences retrieved from the soils formed a distinct clade from known methanotrophs and only showed 80% amino acid identity to their closest relative, *Methylocapsa acidiphila* (also see Fig. 1). This clade of *pmoA* sequences was originally designated RA14 but is now most often referred to as upland soil cluster alpha: USCα (Knief and Dunfield 2005).

With no cultured representatives, insights into the genomic and metabolic potential of the USCα clade were previously limited to a 42-kb fosmid clone that contained the key genes for methane oxidation (Ricke et al. 2005). Recently, however, targeted cell enrichments of an acidic (pH ~4) forest soil in Germany combined with metagenomic analyses have allowed for the reconstruction of a draft genome of a USCα clade member (Pratscher et al. 2018). Binning of multiple metagenomes based on tetranucleotide frequency and abundance identified a USCα bin that also contained a partial 16S rRNA gene sequence. The 16S-rRNA sequence showed 96% sequence identity to *Methylocapsa palsarum* NE2 and *Methylocapsa aurea* KYG, the two most closely related cultivated strains. In agreement with previous findings, the *pmoA* sequence also clustered with sequences from *Methylocapsa*, suggesting a close evolutionary relationship between these groups. In analyzing the reconstructed genome, Pratscher et al. (2018) identified only a single

pmoCAB operon, no sMMO and proposed that carbon assimilation occurs primarily via the serine cycle. Enzymes supporting acetate utilization via the glyoxylate cycle were further identified in the draft genome, suggesting members of USCα may be facultative methanotrophs.

Multiple alphaproteobacterial methanotrophs have now been isolated from forest soil (Table 1). One of the first was *Methylocella silvestris* BL2, which was isolated from an acidic (pH ~4.0) forest cambisol near Marburg, Germany. Like other *Methylocella*, this bacterium lacks intracellular membranes and a pMMO but encodes an sMMO (Dunfield et al. 2003). It is also capable of facultative growth as it grows on methylamine, acetate, pyruvate, succinate, malate, ethanol, and methanol in addition to methane (Dedysh et al. 2005). Two other strains, *Methylocapsa aurea* KYG and *Methyloferula stellata* LAY, were also isolated from acidic forest soils in Germany (Dunfield et al. 2010; Vorobev et al. 2011). Like *Methylocella silvestris* BL2, *Methylocapsa aurea* KYG is a facultative methanotroph capable of weak growth on acetate (Dunfield et al. 2010). Unlike *Methylocella* strains, however, *Methylocapsa aurea* KYG has a pMMO and intracellular membranes, but no sMMO. *Methyloferula stellata* LAY has only an sMMO. The three strains can be further differentiated by their pH optima which are 5.5, 6.0–6.2, and 4.8–5.2 for *Methylocella silvestris*, *Methylocapsa aurea,* and *Methyloferula stellata,* respectively.

One of the first gammaproteobacterial methanotrophs isolated from forest soil was *Methylovulum miyakonense* HT12 (Iguchi et al. 2010). Isolated from a forest soil in Japan, this methanotroph is neutrophilic with a growth range of pH 6.0–7.5. An obligate methanotroph, *Methylovulum miyakonense,* has both particulate and soluble versions of MMO (Iguchi et al. 2010; Hamilton et al. 2015) and assimilates carbon via the ribulose monophosphate pathway (Table 1). The bacterium also lacks unsaturated C_{16}-fatty acids, which are typical of most type-I methanotrophs.

Two distinct bacterial strains, BFH1 and BFH2, have also been isolated from tropical topsoil in Bangladesh (Islam et al. 2016). Both strains are capable of growth at pH 4.2–7.5 and can grow at temperatures up to 60 °C. Genes for *pmoA* were detected, but *mmoX* was absent. Analysis of the 16S gene sequence suggests that these isolates represent a novel genus in the family *Methylococcaceae*, but these strains have not yet been described taxonomically.

5 Future Prospects and Research Needs

Conventional and omic-technologies have provided insights into community structure-function relationships in acidophilic, methane-consuming environments. Continued research into (i) the relative contribution of different species to methane uptake and (ii) a better understanding of the factors that control dominance by distinct lineages is warranted. A comparative analysis of the methanotrophs in tropical peatlands would also be valuable to compare with the large number of studies undertaken in northern latitudes.

There is a particular need to elucidate the anaerobic methane-oxidizers. A lack of cultivated representatives capable of AOM has hindered physiological analyses of this group as well as an accurate assessment of their methane-uptake potential. While mechanisms for AOM have begun to emerge (Table 2), many of these require additional confirmatory evidence made possible only through axenic-culture experimentation. Improved understanding of the potential for environmentally relevant, non-O_2 electron acceptors (or shuttles such as humic acids in peat) to contribute to methane oxidation would be of tremendous value in understanding acidophilic methane consumption through AOM-processes.

Two consistent themes emerge in efforts to better understand global methanotrophic processes, particularly in regards to acidophilic methanotrophy. These include the continued exploration of the diversity of methanotrophic bacteria and an improved understanding of the factors that limit niche expansion. For example, the study by Sharp et al. (2014) provided an assessment of the distribution of verrucomicrobial methanotrophs in Canada and New Zealand. Expansion of this study to other regions would be of value and could possibly provide insights into why verrucomicrobial methanotrophs seem limited to geothermally-influenced soils. Similarly, the factors limiting cultivation of USCα members remain unknown. Continued exploration and technological advancements may soon provide novel strategies for cultivation, but insights into the metabolic potential of this globally important group will remain somewhat elusive until this can be achieved. By developing a better understanding of the phylogenetic and physiological diversity of acidophilic methanotrophs, it will allow for improved understanding of how these environments contribute to the global methane cycle now and in the future.

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