41 Acidic Environments

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Abstract: Methane oxidation has been measured in several acidic environments, including peatlands, forest soils, and terrestrial geothermal features. Aerobic methanotrophic bacteria have been detected in each of these environments using molecular cultivation-independent methods, and anaerobic methanotrophs are also suspected to be active in peat. Most known methanotrophs are neutrophilic, but moderately acidophilic alphaproteobacterial methanotrophs have been isolated and characterized. These grow between pH 4.2 and 7.5, and appear to be the predominant methane-oxidizing species in acidic peatlands and forest soils. Extremely acidophilic methanotrophs able to grow below pH 1 and belonging to the phylum *Verrucomicrobia* have also been isolated from geothermal habitats. This chapter outlines the ecology, physiology, taxonomy, and genetics of methanotrophs in acidic environments.

1 Introduction

Methanotrophic microorganisms are present and are active in environments with pH values ranging from below 2 to above 10. Acidic environments that have active methanotrophic communities include peatlands, forest soils, and geothermal environments. Acidophilic methanotrophs have been isolated from two main bacterial taxa: the phylum *Verrucomicrobia* and the class *Alphaproteobacteria* of the phylum *Proteobacteria*. Characteristics of these species are compared in ❯ *Table 1*. Methanotrophic *Gammaproteobacteria* have also been detected in some mildly acidic environments, but none have been isolated to date. This chapter outlines the ecology, physiology, taxonomy, and genetics of methanotrophs found in acidic environments.

2 Peatlands

Peatlands are oligotrophic environments with pH values ranging from 3.5 to 7, but typically below 5. They account for an estimated 20–30% of the global soil carbon pool and are a major source of atmospheric methane (Gorham, 1991). The vast expanse of northern peatlands, primarily *Sphagnum* bogs and tundra in Russia, Canada, and Alaska, are important particularly because of the potential feedback effects of global warming on methane and carbon dioxide emissions from these habitats (ACIA, 2004).

Aerobic methanotrophic bacteria live in surface layers of wetlands and consume 10–90% of the methane produced in deeper anaerobic zones before it reaches the atmosphere (Segers, 1998). Methanotrophic activity in various Canadian peats was observed to have an acidic optimum (pH 5–6) although this was 1–2 pH units higher than the native peat pH (Dunfield et al., 1993). Three major aerobic methanotroph populations are found in peats, belonging to the proteobacterial class *Alphaproteobacteria*: *Methylocella* spp., *Methylocapsa* spp., and *Methylocystis* spp. (❯ *Table 1*). Three types of data indicate that these are predominant: (1) cultivation studies, (2) enumeration using species-specific oligonucleotide probes in fluorescent in situ hybridization (FISH), and (3) recovery of methanotroph-specific (*pmoA* and *mmoX*) genes from the environment. The *pmoA* gene codes for the active site-containing subunit of particulate methane monooxygenase (pMMO). The gene is an excellent phylogenetic marker because it is universal to all known aerobic methanotrophs except *Methylocella* spp., and because phylogenies constructed based on *pmoA* sequences closely correspond to 16S rRNA gene-based phylogenies. Cultivation-independent recovery of *pmoA* and comparative

Table 1

Comparison of all acidophilic methanotrophic isolates

Species	*Methylocapsa acidiphila*	*Methylocella* spp. (*palustris, silvestris, tundrae*)	*Methylocystis heyeri*	*Methylacidiphilum* spp.
Affiliation	*Alphaproteobacteria*	*Alphaproteobacteria*	*Alphaproteobacteria*	*Verrucomicrobia*
Type strain	B2 (DSM 13967, NCIMB 13765)	K (*M. palustris*) (ATCC 700799)	H2 (DSM 16984, VKM B-2426)	V4
Internal membranes or compartments	Type III: a single membrane stack along one side of the cell envelope	Vesicular membrane invaginations connected to the cytoplasmic membrane	Type II: paired membrane stacks along the cell periphery, parallel to the envelope	Carboxysome-like structures, tubular membranes observed in rare cells
pH optimum	5.0–5.5	5.0–5.5 (*palustris*), 5.5 (*silvestris*), 5.5–6.0 (*tundrae*)	5.8–6.2	2.0–3.5
pH range	4.2–7.2	4.2–7.5	4.4–7.5	0.8–6.0
Major PLFAs (more than 5% of total)	18: 1ω7c (78%), 18: 0 (8%), 16: 0 (7%), 16: 1ω7c (5%)	18: 1ω7c (59–83%), 19: 0ω8c cyclo (0–14%),16: 1ω7c (7–11%), 16: 0 (3–8%), 17: 0 cyclo (0–6.5%), 16: 1ω7t (0–6%)	18: 1ω8c (32–33%), 16: 1ω8c (25–29%) 18: 1ω9t (2–15%), 18: 1ω7c (11–13%), 16: 1ω7c (3–11%), 16: 0 (1–7%)	18: 0 (39%), 16: 0 (14%), 15: 0 (13%), 14: 0 (13%), i14: 0 (7%), 17: 0 (6%)
pMMO	+	–	+	+
sMMO	–	+	+	–
Carbon fixation pathway	Serine cycle	Serine cycle, Calvin-Benson-Bassham cycle[a]	Serine cycle	Calvin-Benson-Bassham cycle[a], Serine cycle[b]
Habitats isolated from (pH in situ)	*Sphagnum-Carex* peat bog (3.6–4.5)	Peat bog (3.6–4.5), temperate soil (3.8–4.3), tundra (5.5–5.8)	Tropical soil (4.2), peat bog lake Teufelsee (4.3)	Thermal mudpot (1–2), Thermal soil (3.1–4.7), Thermal spring (3)
Temperature range in °C (optimum)	10–30 (20)	10–28 (15–20) *palustris*, 4–30 (15–25) *silvestris*, 5–30 (15) *tundrae*	5–30 (25)	37–65 (55–60)

Table 1 (Continued)

Dimensions (μm)	0.7–1.0 by 0.8–1.2	0.6–1.0 by 1.0–2.5	0.8–1.2 by 1.4–4.0	0.3–0.65 by 0.8–4.0
Resting stages	*Azotobacter*-type cysts	Unknown	Lipid cysts	Unknown
N fixation	+	+	+	+[a]
Obligate methylotroph	+	−	+	+
Other features	Salt-sensitive	Salt-sensitive, poly-β-hydroxybutyrate granules at each pole	Poly-β-hydroxybutyrate granules	Carboxysome-like structures
Exopoly-saccharide capsule	+	+(*silvestris* and *palustris*) -(*tundrae*)	+ extensive	−

[a]presence based on genome prediction only
[b]a variant of the serine cycle appears to operate, using glyoxylate shunt enzymes to regenerate glyoxylate

sequence analysis is therefore the most popular method of characterizing methanotrophic communities in natural environments (Dumont and Murrell, 2005). The *mmoX* gene encodes a subunit of soluble methane monooxygenase (sMMO). This enzyme is not universal to methanotrophs, but unlike *pmoA* it can be used to target *Methylocella* in molecular ecology studies.

2.1 Methylocella

Methylocella is treated in detail in another chapter of this volume, and therefore will be described superficially. This alphaproteobacterium has many unique features compared to other methanotrophs (Table 1). Notably, it lacks an extensive intracellular membrane system, uses only sMMO for methane oxidation, and is able to grow on some substrates containing C–C bonds. *Methylocella* is a moderate acidophile found in diverse habitats, especially peatlands and acidic soils.

2.2 Methylocapsa

Methylocapsa acidiphila (Dedysh et al., 2002) is closely related (96–97% 16S rRNA gene sequence identity) to *Methylocella*. *Methylocapsa* and *Methylocella* have a similar mildly acidophilic phenotype, but *Methylocapsa* is an obligate methanotroph with a pMMO enzyme and an extensive intracellular membrane system, whereas *Methylocella* is a facultative methanotroph with an sMMO and a vesicular membrane system (Table 1, Fig. 1). *Methylocapsa* was isolated from a *Sphagnum* bog in Siberia. Some *pmoA* sequences closely related to it have also been recovered from mildly acidic soil (pH 5.8–6.5) (Horz et al., 2002).

Although *Methylocella* and *Methylocapsa* spp. are expected to be free-living bacteria, phylogenetically closely related bacteria (93% 16S rRNA gene sequence identity) have also been found in close association with *Sphagnum* plants (Raghoebarsing et al., 2005). Using 16S-rRNA targeted FISH probes, these bacteria were observed within hyaline cells (water-filled, dead, porous cells) of the outer stem cortex and on the surface of stem leaves.

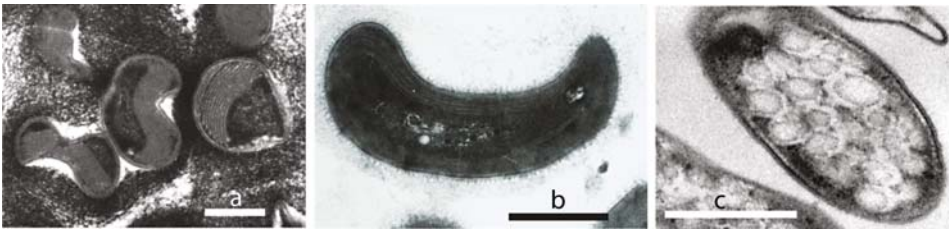

Figure 1

Electron micrographs of acidophilic methanotrophs: (a) *Methylocapsa acidiphila*, (b): *Methylocystis heyeri*; (c) *Verrucomicrobia* isolate V4 (*Methylacidiphilum infernorum*). The scale bar is 0.5 μm.

2.3 Methylocystis

Methylocystis spp. are found in diverse methane-rich habitats (Heyer et al., 2002). These are *Alphaproteobacteria*, traditionally designated along with the genus *Methylosinus* as "Type II" methanotrophs. Many strains have sMMO in addition to pMMO enzymes, and all have intracellular membranes (Fig. 1). Most are neutrophilic, but one moderate acidophile, *Methylocystis heyeri*, was isolated from peat and forest soils (Dedysh et al., 2007). The *pmoA* sequence of this organism is similar to a distinct subgroup of *pmoA* sequences frequently detected by cultivation-independent methods in peat soils (Fig. 2).

2.4 Ecological Studies

Cultivation-independent molecular recovery of *pmoA* and *mmoX* genes from peatlands has repeatedly demonstrated the presence of *Methylocystis* (Fig. 2) (Chen et al., 2008; Jaatinen et al., 2005; McDonald et al., 1997; Morris et al., 2002). In addition, recovery of *pmoA* mRNA

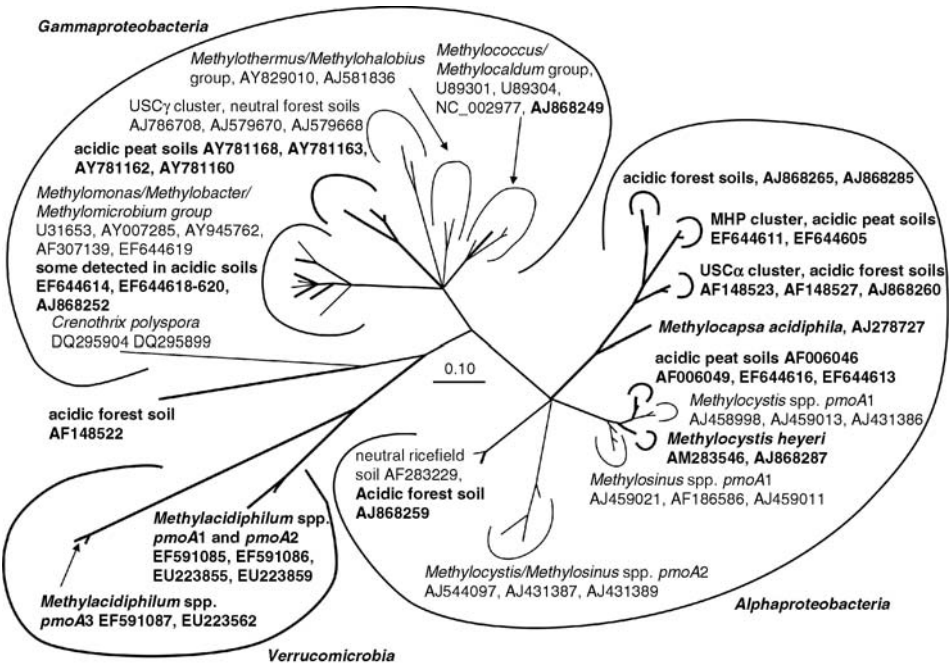

Figure 2

Phylogenetic tree constructed based on partial (495 positions) *pmoA* gene sequences, comparing reference sequences to acidophilic methanotrophic isolates and to some genes detected in acidic soil environments (pH <5.2) by Chen et al. (2008), Holmes et al. (1999), Jaatinen et al. (2005), Knief et al. (2005), Knief et al. (2006), and McDonald and Murrell (1997). All acidophilic isolates and sequences detected in acidic environments are in bold. The tree was constructed using Treepuzzle, a quartet maximum likelihood method, using the Schöniger-von Haeseler distance correction method (Schmidt et al., 2002). Nodes are supported by >50% based on 5,000 puzzling steps. Where the support value is <50% a multifurcation is drawn. The scale bar represents 0.1 change per position.

transcripts of *Methylocystis* from Moor House Peat in the UK suggested that these bacteria were actively consuming methane (Chen et al., 2008). Although *Methylocapsa acidiphila* has not been detected by these methods, Chen et al. (2008) detected a predominant group ("MHP" clade) of *pmoA* sequences somewhat related to *Methylocapsa* in *Calluna*-covered moorlands (pH 4.6) of the UK (Fig. 2). *Methylocella* lacks a pMMO enzyme and therefore cannot be detected via *pmoA* recovery, but *Methylocella*-like *mmoX* sequences have been found in UK peat ecosystems (Chen et al., 2008).

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 *Gammaproteobacterial. 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) were abundant, but *Gammaproteobacteria* accounted for <1% of the methanotroph populations (Dedysh et al., 2001, 2003). Detection and quantification of a "signature" PLFA of *gammaproteobacterial* methanotrophs, C16:1ω8c, has previously been used as evidence that these bacteria are abundant in peatlands (Krumholz et al., 1995; Sundh et al., 1995). However, the recent 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). Likely, detection of this lipid in the above studies resulted from the abundant *Methylocystis* population.

Although quantitative studies suggest that methanotrophic *Alphaproteobacteria* are numerically predominant in peatlands, type I methanotrophs (*Gammaproteobacteria*) can also be detected by FISH (Dedysh et al., 2001, 2003) and by *pmoA* recovery (Fig. 2). *Methylococcus*-like *pmoA* sequences were recovered from a raised mire in Finland (Jaatinen et al., 2005), and *Methylobacter* were detected in heather moorlands and *Sphagnum/Eriophorum* peatlands in the UK (pH 4.3–4.6) using a *pmoA* microarray (Chen et al., 2008). Acidophilic methanotrophic *Gammaproteobacteria* therefore probably exist, but have so far evaded isolation.

2.5 Anaerobic Methane Oxidation in Peat

There is now evidence that anaerobic methane oxidation occurs in some peats, at a similar net rate as aerobic methane oxidation. Smemo and Yavitt (2007) identified the process in several different peat samples by examining: (1) methane depletion in anoxic incubations of peat after addition of methanogenesis inhibitors, (2) stable isotope ($^{13}CH_4$) dilution in anoxic peat incubations, and (3) natural $^{13}CH_4$ abundances. Unlike anaerobic methane oxidation in marine environments, sulfate does not appear to be the major electron acceptor for the process in peatlands. Instead, organic molecules or nitrate may be the major electron acceptors.

3 Geothermal Environments

The CH_4 molar fraction of geothermal gas is typically 0.01–1% (Etiope and Klusman, 2002), but can be much higher. For example, many New Zealand geothermal systems contain 1–11% methane, with anomalies up to 27% (Giggenbach, 1995). An estimated 2.5–6.3 Tg y^{-1} of

methane is emitted annually from geothermal sources to the atmosphere (Etiope and Klusman, 2002). This methane is usually isotopically heavy ($\delta^{13}C$ of −25 to −35‰), and therefore not derived from a microbial source. It is formed primarily via two processes: thermogenic decomposition of buried organic matter, and abiotic Fischer-Tropsch type reactions (Etiope and Klusman, 2002; Giggenbach, 1995). Geothermal environments are characterized by high temperatures and frequently by extreme acidity due to the oxidation of sulfur compounds. Castaldi and Tedesco (2005) measured methane flux in the crater of the Sulfatara volcano in Italy. Methane oxidation potential was detected in laboratory incubations of bare fumarolic soil at pH 1.8 and 70°C. This study presented the first convincing evidence for the existence of extremely acidophilic methanotrophs. By measuring soil gas profiles in a steaming geothermal soil in New Zealand, Dunfield et al. (2007) later demonstrated that methanotrophic bacteria in the surface soil were an effective filter against emission of geothermal methane to the atmosphere.

In 2007, extremely acidophilic methanotrophic bacteria were simultaneously isolated from three acidic geothermal sites: a mudpot in the Solfatara volcano in Italy (isolate SolV), a steaming soil at Tikitere, New Zealand (isolate V4), and an acidic hot spring in Kamchatka, Russia (isolate Kam1) (Dunfield et al., 2007; Islam et al., 2008; Pol et al., 2007). These three bacteria were phylogenetically closely related (<2% 16S rRNA sequence divergence), and together represented the first known methanotrophs from a bacterial phylum other than the *Proteobacteria*. They belonged to the *Verrucomicrobia*, an abundant, diverse, and widespread group of bacteria that is represented by only a handful of isolates in culture collections. They are presently being described as the genus *Methylacidiphilum* (unpublished data). As expected, these isolates grow under hot, acidic conditions (*Table 1*). Their pH optimum for methane oxidation and growth is 2.0–3.5, and the lower limit is 0.8. Membrane permeability to protons is minimized by having phospholipids that are almost entirely saturated, in stark contrast to the moderately acidophilic proteobacterial methanotrophs (*Table 1*). Genome analysis suggests that key elements of thermoacidophilic adaptation include a variety of ion transporters, such as an Na^+/H^+ antiporter that could convert the proton motive force to a sodium motive force (Hou et al., 2008). Other pH regulatory mechanisms are present to bind excess H^+ ions in the cytoplasm, and the distribution of isoelectric points of proteins determined in silico from genome data shows a large excess of high-pI (basic) proteins (Hou et al., 2008).

Besides their extremely acidophilic phenotype, methanotrophic *Verrucomicrobia* have several other unique features compared to their *Proteobacteria* counterparts (*Table 1*). Internal membrane systems are missing. Instead there are carboxysome-like compartments in cells of all three isolates (*Fig. 1*). Draft genomic analyses were made for the *Verrucomicrobial* methanotrophs V4 and SolV (Dunfield et al., 2007; Pol et al., 2007), and a full genome analyses of V4 followed (Hou et al., 2008). These detected three paralogous *pmo* operons encoding pMMO in each bacterium (*Fig. 2*). The three paralogs differed by as much as 50% in amino acid content, and phylogenetic analyses suggested that they originated from two lineage-specific duplications. The V4 genome also encodes a complete Calvin-Benson-Bassham cycle. Carbon fixation could therefore be partially autotrophic, however a variant of the serine cycle for C fixation from formaldehyde may also be present. This is similar to the serine cycle in *Alphaproteobacteria* methanotrophs, except that glyoxylate is regenerated using the glyoxylate shunt enzymes isocitrate lyase and malate synthase. The *Verrucomicrobial* methanotrophs also lack the tetrahydromethanopterin pathway for C1 transfer that is a common property of other methylotrophs.

4 Acidic Forest Soils

Aerobic upland soils, particularly forest soils, constitute a net methane sink that consumes an estimated 7–100 Tg of atmospheric CH_4 per year (reviewed in Dunfield, 2006). Methanotrophic bacteria in these soils oxidize methane from the overlying atmosphere, where it is present at a mixing ratio of about 1.75 ppmv. Methane uptake in these soils displays a typical Michaelis-Menten response to methane concentration, but the apparent affinity for methane is several orders of magnitude higher (10–100 nM) than that observed in methanotrophic cultures (1–10 μM). This suggests that methanotrophs in upland soils are oligotrophic and possess a high-affinity form of MMO to survive on the trace level of atmospheric methane. These bacteria are usually called "high-affinity methane oxidizers" or "atmospheric methane oxidizers," but have not yet been isolated into pure culture (Dunfield, 2006).

Atmospheric methane consumption has been measured in soils with pH values ranging from 3 to 8 (e.g., Knief et al., 2003), but most forest soils are moderately acidic (pH 4–6). It is therefore expected that methanotrophs in these soils are acidophiles as well as oligotrophs. Amaral et al. (1998) observed that methanotrophic bacteria extracted from two acidic (pH 4.5–5.2) forest soils by high-speed blending and density gradient centrifugation were slightly acidophilic. The pH optimum for methanotrophy was 5.8 and no activity was detected at pH 6.8.

Holmes et al. (1999) characterized atmospheric methane-oxidizing bacteria in three acidic forest soils (pH 3.4–4.9) using a dual approach of cultivation-independent retrieval of *pmoA* genes and radiolabeling of microbial phospholipid fatty acids (PLFAs) by incubation of soil under a low mixing ratio (<50 ppmv) of $^{14}CH_4$. The ^{14}C-labelled PLFAs recovered were similar to PLFAs of acidophilic alphaproteobacterial methanotrophs, particularly *Methylocapsa*. Examination of ^{13}C-labelled PLFAs and bacteriohopanoids detected after incubation of acidic forest soils under ^{13}C-labelled methane has supported the theory that methanotrophs related to *Methylocapsa* are present and active in acidic soils (Crossman et al., 2005; Knief et al., 2003). Based on sequence phylogeny, the *pmoA* gene sequences detected by Holmes et al. (1999) belonged predominantly to a cluster not identical to the *pmoA* of any known methanotroph, but most closely related (80% amino acid identity) to *Methylocapsa acidiphila*. This cluster, usually called USCα (Fig. 2), was recovered from many different acidic upland soils in subsequent studies (Dunfield, 2006). Kolb et al. (2005) designed quantitative real-time PCR assays targeting specific phylogenetic groups of *pmoA* genes, and demonstrated that USCα was by far the most abundant *pmoA* sequence detectable in an acidic (pH 4.3) forest soil, at 0.9–2.1 × 10^6 copies g^{-1} soil. However, this predominance is limited only to acidic soils. Using a large set of soils ranging from pH 3.9–8.0, Knief et al. (2003) demonstrated that USCα was predominantly detected in acidic soils (<pH 6), but a separate group of *pmoA* sequences related to *Gammaproteobacteria* (USCγ) was predominantly detected in soils with pH > 6. This supports the assumption that the USCα represents an acidophilic methanotroph.

Ricke et al. (2005) screened a fosmid library of 250,000 clones prepared from a soil DNA extract using *pmoA*-targeted PCR, and found a 42-kb genomic contig containing the *pmoA* gene of a USCα bacterium. The close taxonomic relationship of USCα to *Methylocapsa* was verified by the following analyses of the contig: (1) tetranucleotide frequency patterns of 5-kb genomic subfragments, (2) annotation and comparative analysis of the genomic fragments against completely sequenced genomes, and (3) phylogenies constructed using three other genes located on the contig.

5 Prospects and Research Needs

Complete genome sequences for *Methylocapsa acidiphila* and *Methylocella silvestris* are presently being generated by the Joint Genome Institute of the US Department of Energy. Analysis of these genomes, and comparison to other methanotrophs such as *Methylococcus capsulatus* and the methanotrophic *Verrucomicrobia*, should shed more light on the physiology and genetics of acidophilic methanotrophs.

Ecologically, a priority is determining how different groups are distributed in different environments. A question of particular interest is whether the *Verrucomicrobia* methanotrophs are confined to geothermal habitats. Because of primer region mismatches with the *pmoA* oligonucleotide primers used in most PCR-based ecological studies, these bacteria cannot be detected using standard *pmoA* assays. They may therefore have been overlooked in previous studies of various environments. A methanotrophic isolate from the phylum *Verrucomicrobia* has been grown at 25°C (Dunfield et al., 2007), so this group of is probably not limited to thermophiles. Morris et al. (2002) recovered one 16S rRNA gene sequence affiliated with a verrucomicrobium in a $^{13}CH_4$ SIP experiment using (pH-neutral) peat. This could have resulted from cross-feeding (e.g., via $^{13}CO_2$), but may also have resulted from direct consumption of $^{13}CH_4$.

Finally, a variety of other *pmoA* sequences that cannot be affiliated to known genera of methanotrophs have been recovered in cultivation-independent assays of acidic environments, especially soils (◉ *Fig. 2*) (e.g., Holmes et al., 1999; Knief et al., 2006). There appears to be many unknown genera and species that still await isolation and characterization. These may include species in other classes of the *Proteobacteria*, or even other bacterial phyla.

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