Chapter 9
Unusual Members of the PVC Superphylum: The Methanotrophic *Verrucomicrobia* Genus "*Methylacidiphilum*"

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9.1 Introduction

Methane is an important molecule in the environment. From a societal perspective, it is a clean alternative fuel as well as a greenhouse gas contributing 18 % of total atmospheric radiative forcing (IPCC). From an ecological point of view it is a key intermediate in the global carbon cycle. In the absence of primary electron acceptors such as O_2, SO_4^{2-}, and NO_3^-, methanogenic archaea couple the oxidation of small molecules like acetate and H_2 to the reduction of CO_2 or a methyl group to form methane. An estimated 2 Gt of methane is produced each year through anaerobic degradation of organic matter (Thauer et al. 2008). Methane can also be formed

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J.A. Fuerst (ed.), *Planctomycetes: Cell Structure, Origins and Biology*,
DOI 10.1007/978-1-62703-502-6_9,

by nonmicrobial processes in the deep subsurface and efflux to the atmosphere from seeps and geothermal zones.

Methanogenic environments are therefore usually subsurface environments, either shallow flooded sediments for archaeal methanogenesis or deeper thermal zones for geochemical processes. As methane diffuses upwards from its subsurface origins towards surface oxic zones, it becomes an energy source for methanotrophs. These microbes couple the oxidation of methane to the reduction of O_2, SO_4^{2-}, or NO_2^-. The SO_4^{2-}-driven methanotrophic consortia are particularly important in ocean seeps and methane hydrate deposits (Knittel and Boetius 2009). The only known NO_2^--using methanotroph belongs to the bacterial candidate phylum NC10 and has been tentatively named "*Candidatus Methylomirabilis oxyfera*" (Ettwig et al. 2009, 2010). It was demonstrated that this bacterium has the unique ability to produce intracellular O_2 through an alternative denitrification pathway (Ettwig et al. 2010). The importance of this process in the environment is currently being investigated. However, of the three physiological types of methanotrophs, the O_2-using or aerobic methanotrophs are the best-studied group.

The taxonomic and ecological diversity of aerobic methanotrophs was once thought to be extremely limited. Until recently all known species belonged to the bacterial phylum *Proteobacteria*, specifically to the classes *Gammaproteobacteria* and *Alphaproteobacteria*. It was considered that gammaproteobacterial and alphaproteobacterial methanotrophs could be easily distinguished on the basis of a few physiological and biochemical characteristics (Hanson and Hanson 1996), and they were therefore often treated as two coherent groups called type I and type II methanotrophs, respectively. However with ongoing study the known physiological and ecological diversity within each group has expanded. There are now 18 described genera of proteobacterial methanotrophs (Table 9.1), and many do not conform to the simple characteristics once used to delineate the two types (Op den Camp et al. 2009; Stein et al. 2012). Even more interesting from a biodiversity perspective is that non-proteobacterial methanotrophs have now also been found. These include some members of the phylum *Verrucomicrobia* (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008) and a member of the candidate division NC10 (Ettwig et al. 2009, 2010).

9.2 Methanotrophic *Verrucomicrobia*

Methanotrophic *Verrucomicrobia* have to date only been found in geothermal environments. Geothermal gas typically contains 0.1–1 % CH_4, with a $\delta^{13}C$ signature of −20 ‰ to −30 ‰, strongly indicating a nonmicrobial source (Etiope and Klusman 2002; Giggenbach 1994). This nonmicrobial source may be thermogenic, in which methane is formed from the thermal cracking of buried organic matter, or abiotic, in which H_2 and CO_2 react to form methane in Fischer-Tropsch-type reactions (Xu 2010). Methane is emitted from geothermal systems via degassing of spring water and diffuse gas seepage from underground reservoirs through soil cover. As in non-geothermal systems this release may be constrained by the activity of

Table 9.1 Comparison of the three main phylogenetic groups of aerobic methanotrophic bacteria

Phylum and class	*Proteobacteria* (*Gammaproteobacteria*)	*Proteobacteria* (*Alphaproteobacteria*)	*Verrucomicrobia* subphylum 6
Genera	*Methylococcus, Methylocaldum, Methylohalobius, Methylothermus, Methylobacter, Methylomicrobium, Methylomonas, Methylosarcina, Methylosoma, Crenothrix, Clonothrix, Methylosphaera, Methylovulum*	*Methylocystis, Methylosinus, Methylocella, Methylocapsa, Methyloferula*	"*Methylacidiphilum*"
Internal membranes or compartments	Type I: membrane bundles perpendicular to the cell envelope	Type II: membrane stacks along the cell periphery, parallel to the cell envelope (except *Methylocella*)	Uncharacterized inclusion bodies
Lowest reported growth pH	5.0 (many species)	4.2 (*Methylocapsa acidiphila*)	0.8 (*M. fumariolicum* SolV)
Highest reported growth pH	11 (*Methylomicrobium buryatense*)	9.5 (*Methylocystis* sp. strain B3)	6.0 (*M. infernorum* V4)
Lowest reported growth T (°C)	0 (*Methylosphaera hansonii*)	4 (*Methylocella silvestris*)	37 (*M. kamchatkense* Kam1)
Highest reported growth T (°C)	72 (*Methylothermus* sp. strain HB)	40 (many species)	65 (*M. fumariolicum* SolV)
Major PLFAs (more than 15 % of total in any species)	14:0 (1–24 %) 16:1ω8c (0–41 %) 16:0 (4–63 %) 18:1ω7c (0–60 %) 16:1ω5t (0–30 %)⋯ 18:1ω9c (0–35 %) 16:1ω7c (8–57 %)	16:1ω8c (0–29 %) 18:1ω7c (10–83 %) 18:1ω8c (32–74 %)	i14:0 (7–22 %) a15:0 (13–31 %) 18:0 (14–42 %)
Carbon fixation pathway	Ribulose monophosphate pathway, Calvin-Benson-Bassham cycle	Serine cycle, Calvin-Benson-Bassham cycle	Calvin-Benson-Bassham cycle
G+C mol%	43–65	60–67	40.8–45.5
N_2 fixation	±	±	±
sMMO	±	±	−
pMMO	+	±	+

For detailed references see Op den Camp et al. (2009) and Dunfield (2009)

methanotrophic bacteria. In 2005, aerobic methane oxidation activity was reported in laboratory incubations of soils from the Solfatara volcano (Italy) with pH levels as low as 1.8 and temperatures as high as 70 °C (Castaldi and Tedesco 2005). A later study of steaming soil in Hell's Gate (Tikitere), New Zealand (pH 3–5, 60 °C), suggested that methanotrophic bacteria at this site were also actively consuming methane in geothermal gas (Dunfield et al. 2007). The acidic conditions in these environments were too harsh to allow the survival of any known methanotroph.

The microorganisms responsible for methane oxidation in extremely acidic geothermal environments were identified in three independent publications in 2007–2008 (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008). Remarkably, they were not alpha- or gammaproteobacteria but rather members of a distinct phylum within the domain *Bacteria*, the *Verrucomicrobia*. The three isolates described probably represent a single genus based on the high level of similarity among their 16S ribosomal RNA (rRNA) gene sequences (>98.4 %). There have been difficulties in getting these bacteria preserved in a viable state in public culture collections, and therefore, they are still not taxonomically validated. However, Op den Camp et al. (2009) suggested that they be all considered members of a single genus called "*Methylacidiphilum*," with three proposed species: "*M. kamchatkense*" Kam1, "*M. infernorum*" V4, and "*M. fumariolicum*" SolV. The three isolates of "*Methylacidiphilum*" are remarkably similar (see comparison table in Op den Camp et al. 2009), and they will be treated as a single entity in this chapter.

Hedlund (2010) classifies all 16S rRNA sequences from cultured and uncultured *Verrucomicrobia* into 7 subphyla or classes, of which only three are named: *Verrucomicrobiae, Opitutae*, and "*Spartobacteria*." "*Methylacidiphilum*" is the only cultured representative of subphylum 6 (Fig. 9.1). Most sequences in this group have been recovered from geothermal habitats, although a few are from soils or invertebrate guts (Hedlund 2010). It is unknown whether other bacteria in subphylum 6 are methanotrophic.

Published physiological studies of "*Methylacidiphilum*" are still quite few. However, a complete genome is available for strain V4 (Hou et al. 2008) and draft genomes for SolV (Khadem et al. 2012a) and Kam 1 (unpublished). These have allowed detailed comparisons of metabolic potential and evolutionary histories to be made to model proteobacterial methanotrophs, for which several genomes are now available. An interesting element of the genome analysis of strain V4 by Hou et al. (2008) was a correspondence analysis of its gene content compared to 58 other bacteria. Orthologous groups were defined based on the eggNOG database. This analysis suggested that the gene content of V4 was more similar to species of *Proteobacteria* than to other *Verrucomicrobia*. This may indicate a history of promiscuous lateral gene transfer (LGT). However, given that the analysis was more a comparison of metabolic lifestyle than a phylogenetic analysis, this conclusion is not certain. Based on the closest BLAST hits, the JGI IMG site for strain V4 predicts 970 genes (38.5 %) as putatively laterally transferred. Again, this is likely to some extent an artifact of the few verrucomicrobial genomes in the database (9), but it is also suggestive of a fluid genome. Alien Hunter, a program that uses nucleotide dimer to octamer frequencies to identify large islands of LGT (Vernikos and Parkhill 2006),

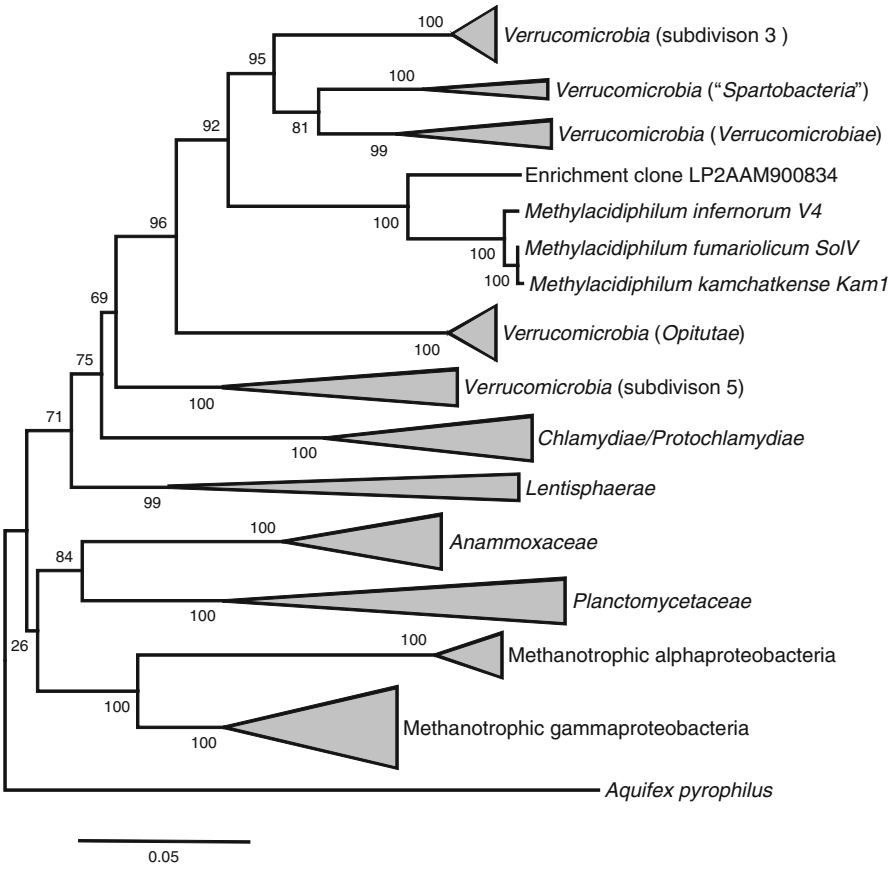

Fig. 9.1 Neighbor-joining 16S rRNA gene-based phylogeny showing 6 of the 7 main subdivisions of *Verrucomicrobia,* including "*Methylacidiphilum*" in subdivision 6, in relation to other phyla of the domain *Bacteria.* The other aerobic methanotrophs within the phylum *Proteobacteria* are included for reference. Node support values are based on 500 bootstrap replicates. Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007). The scale bar represents 0.05 changes per nucleotide position

identifies 29 regions of the V4 genome as putatively foreign DNA, summing to a total of 270,000 nt (11.80 % of the genome). The largest region is 32,500 nt. This program is conservative in that it can only identify large regions of LGT (5,000 nt windows), as only these provide enough data to estimate octamer frequencies.

A close phylogenetic examination of key methane metabolism genes shows some evidence for lateral transfer. Formate dehydrogenase and formate hydrogen lyase genes associate most closely with genes from *Proteobacteria* and *Aquificae*. Several genes involved in nitrogen metabolism, which are intimately linked to methane oxidation (Stein et al. 2012), show phylogenetic evidence of lateral transfer as well. These include the nitric oxide reductase-encoding genes *norB* and *norC,*

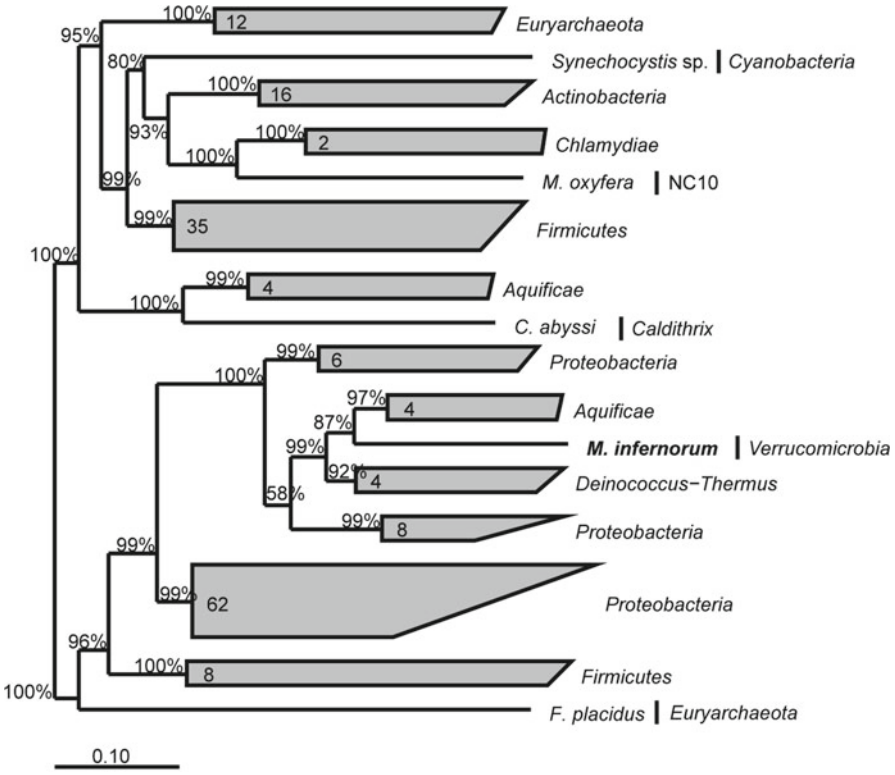

Fig. 9.2 Neighbor-joining phylogenetic tree of 166 derived amino acid sequences (length 474) of nitric oxide reductase subunit B (NorB). The tree was constructed using the ARB software environment (Ludwig et al. 2004). Node support values are based on 5,000 bootstrap replicates. The scale bar represents 0.1 changes per amino acid position

which form a phylogenetic cluster together with sequences from *Aquificae* and *Thermus/Deinococcus,* nested within a large group of genes from *Proteobacteria* (Fig. 9.2). The hydroxylamine oxidoreductase-encoding *haoA* was possibly obtained via LGT from *Proteobacteria* (see the phylogeny in Klotz et al. 2008). Nitrogen fixation genes such as *nifHDK* are most closely related to genes from *Proteobacteria* as well (Khadem et al. 2010), and the entire gene set encoding nitrogen fixation is likely to have been acquired via lateral transfer. *Methylacidiphilum* is also highly unusual in possessing two distinct H^+ pumping F_1F_0 ATPases, one of which appears to be ancestral to *Verrucomicrobia* and the other obtained from a gammaproteobacterium (Hou et al. 2008). We present more evidence of LGT later.

A unique acidic and thermostable bacterial hemoglobin has been characterized from "*Methylacidiphilum infernorum*," named Hell's Gate globin I (HGbl). HGbl is unusual due to an extremely high resistance to oxidation at low pH and high temperature. It also has high structural resemblance to the vertebrate neuroglobins, providing a strong evolutionary link between the bacterial flavohemoglobins and eukaryotic neuroglobins (Teh et al. 2011).

9.3 Physiological Comparison with Proteobacterial Methanotrophs

Species of proteobacterial methanotrophs differ from each other in their dominant phospholipid fatty acids, type of methane monooxygenase (MMO) present, nitrogen fixation ability, geometric arrangement of intracellular membranes, ecological tolerances (to temperature, salt, and acid stresses), genetic systems available to deal with nitrification stress, and carbon assimilation pathways. C-assimilation pathways include the ribulose monophosphate pathway (RuMP), the serine cycle, and the Calvin-Benson-Bassham cycle (Hanson and Hanson 1996). The characteristics that most distinguish the verrucomicrobial genus "*Methylacidiphilum*" from its proteobacterial counterparts are an extremely acidophilic phenotype, a prevalence of saturated fatty acids, a general lack of intracytoplasmic membrane stacks but presence of other inclusion bodies, at least as deduced from thin sections of chemically fixed cells, and an inability to fix C at the level of formaldehyde. Most of the other properties of "*Methylacidiphilum,*" particularly its central enzymatic machinery for processing methane, are common to several proteobacterial methanotrophs.

Most proteobacterial methanotrophs are neutrophilic (pH range 5.5–8.5) and mesophilic (temperature range 20–40 °C), although some moderately acidophilic and some thermophilic isolates have been identified (Dunfield 2009) (Table 9.1). The alphaproteobacteria *Methylocapsa* and *Methylocella* are mild acidophiles growing between pH 4.2 and 7.2. These genera, along with the alphaproteobacterium *Methylocystis* and occasionally some gammaproteobacterial methanotrophs like *Methylomonas*, are commonly detected via cultivation-independent methods in acidic peats at pH 3.5–5.0 (Dunfield and Dedysh 2010; Kip et al. 2011). However, the lower limit of pH 0.8 for "*Methylacidiphilum*" is well below this range. The upper temperature limit for growth of "*Methylacidiphilum*" falls just short of that reported (67 °C) for the most thermophilic proteobacterial methanotroph, *Methylothermus thermalis* (Tsubota et al. 2005).

Proteobacterial methanotrophs contain mainly 14C, 16C, and 18C phospholipid fatty acids (PLFAs), usually monounsaturated. Some are signature compounds found exclusively in proteobacterial methanotrophs and not in any other known organism: C16:1ω8c and C16:1ω5t (usually in the gammaproteobacterial methanotrophs) and C18:1ω8c (usually in the alphaproteobacterial methanotrophs) (Bodelier et al. 2009). The PLFA profiles of "*Methylacidiphilum*" are very different and show distinctive characteristics of extreme acidophiles. They contain predominantly (>96 %) saturated fatty acids i14:0, a15:0, and 18:0, consistent with the minimization of membrane permeability to protons (Op den Camp et al. 2009). This is in contrast to even the moderately acidophilic proteobacterial methanotrophs, which contain predominantly unsaturated PLFAs. However, the bacteriohopanepolyol compounds in "*Methylacidiphilum*" are not unique compared to other methanotrophs. The main bacteriohopanepolyol is aminotriol, which is common to many other bacteria, including *Methylocella palustris* (van Winden et al. 2012).

Most proteobacterial methanotrophs oxidize methane via a membrane-bound particulate methane monooxygenase (pMMO). In order to increase the membrane

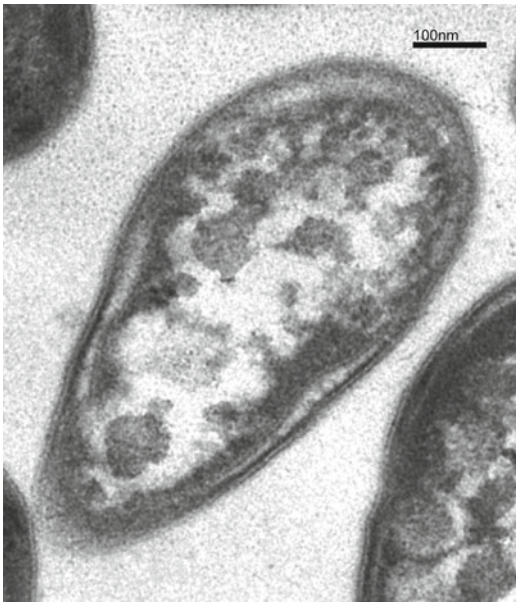

Fig. 9.3 Transmission electron micrograph of a thin-sectioned chemically fixed cell of "*Methylacidiphilum infernorum*" V4 showing a large putative periplasmic space and internal inclusion bodies. Scale bar, 100 nm

surface area to hold this enzyme, they produce internal cytoplasmic membrane stacks. Although "*Methylacidiphilum*" also possesses pMMO, it does not typically display internal membranes. Internal membrane systems were sometimes observed in strain V4 but were not reported in SolV or Kam1. Instead, multiple electron dense inclusion bodies are seen (Fig. 9.3). Although these internal compartments were originally proposed to be carboxysomes, Khadem et al. (2011) determined that the ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) in the SolV genome is a non-carboxysomal type and that carboxysome shell proteins are missing. The exact structure and function of these inclusion bodies has been difficult to resolve as chemical fixation of these bacteria lacks resolution, a phenomenon also seen with members of the *Planctomycetes*. Whether the cells of these strains possess a cell plan like many non-methanotrophic verrucomicrobia and planctomycetes including a major intracytoplasmic membrane also will depend on future application of cryo-techniques alternative to chemical fixation.

9.4 Methane Metabolism

Most methanotrophs are highly specialized. They are incapable of growing on substrates with C-C bonds but rather survive solely on methane, methanol, and a few other 1-carbon compounds (Stein et al. 2012). There are exceptions, notably the facultative methanotroph *Methylocella* (Dedysh et al. 2005). When strain V4 was tested on alternative growth substrates used by *Methylocella,* none supported growth, suggesting it is an obligate methylotroph like most known species

(Dunfield et al. 2007). However, genome data provides interesting clues to a possible facultative metabolism in strain SolV. There is a hydrogenase gene cluster in the draft genome (Khadem et al. 2012a) and the strain was shown to oxidize H_2, although it did not support growth when used as a sole substrate (Pol et al. 2007). Interestingly, the draft genome of SolV also contains acetate kinase and acetyl-coenzyme A synthase (Khadem et al. 2012a), which in theory might confer the ability to grow on acetate. The genome of V4 also contains acetate kinase, but no acetyl-coenzyme A synthase or other phosphotransacetylase could be identified (Hou et al. 2008).

Methanotrophs convert methane to carbon dioxide in the overall reaction: $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$. The first step is catalyzed by the enzyme methane monooxygenase (MMO). In this step, one atom of oxygen is incorporated into methane to generate methanol (CH_3OH). There are two forms of the methane monooxygenase enzyme, a soluble form (sMMO) and particulate form (pMMO). The pMMO form is an enzyme of the copper-containing membrane-bound monooxygenase superfamily that also includes ammonia monooxygenase (AMO). pMMO is universally found in all methanotrophic genera with the exception of *Methylocella,* while the sMMO is found in a few genera. Multiple copies of pMMO genes were detected in each "*Methylacidiphilum*" strain, but no genes for sMMO. Each gene *pmoC*, *pmoA,* and *pmoB* is a distant homologue of its counterpart in proteobacteria, and a similar *pmoCAB* operon structure as in most proteobacterial operons was observed. All "*Methylacidiphilum*" isolates contain three complete *pmoCAB* operons, with V4 possessing an orphan fourth *pmoC* copy and Kam1 an additional *pmoCA* operon (Op den Camp et al. 2009). Interestingly, within each strain the three orthologous operons differ by as much as 50 % at the amino acid level. The most divergent of the three operons is that dubbed *pmoCAB3* (as opposed to *pmoCAB1* and *pmoCAB2*). It has been speculated that each ortholog may have evolved different kinetic properties and thus is differentially expressed under distinct environmental conditions. Baani and Liesack (2008) demonstrated a similar phenomenon in *Methylocystis* sp. strain SC2. This isolate has two copies of the *pmoCAB* operon that exhibit a low degree of amino acid sequence similarity (59.3–65.6 %) between the respective *pmoA*, *pmoB,* and *pmoC* genes of each operon. The two operons have different kinetic affinities to methane and are differentially expressed in response to methane concentration (Baani and Liesack 2008).

Recently, RNA-seq transcriptome analysis of "*M. fumariolicum*" SolV showed that the *pmoCAB*2 operon was highly expressed in cells growing at μmax with excess oxygen and ammonium (Khadem et al. 2012b). The other two *pmoCAB* operons were hardly expressed under these culture conditions. The expression pattern of cells from CH_4-limited, N_2-fixing, and O_2-limited chemostat cultures (dO_2 of 0.5 % and 0.03 % oxygen saturation, respectively) was remarkably different. Under these conditions, the *pmoCAB*2 operon was downregulated 40 times, while the *pmoCAB*1 operon was highly upregulated. The *pmoCAB*3 operon was hardly expressed in these cells. The results point to a regulation of the *pmoCAB*1/*pmoCAB*2 expression by oxygen concentration. In addition, qPCR analysis of the four *pmoA*

genes of "*Methylacidiphilum kamchatkense*" Kam1 showed that the *pmoA*2 gene was 35-fold more strongly expressed than the other copies during batch cultivation (Erikstad et al. 2012). Suboptimal pH and temperature during growth did not change this pattern, but growth on methanol resulted in a tenfold decreased expression of *pmoA*2.

9.5 Evolutionary History of *pmo*

Phylogenetic analysis of all three *pmoA* gene copies relative to *pmoA/amoA* genes of other prokaryotes revealed that all *pmoA* genes from *Verrucomicrobia* fell into a monophyletic cluster, suggesting that the three orthologues are derived via lineage-specific duplication rather than lateral gene transfer (Dunfield et al. 2007) (Fig. 9.4). Phylogenetic analysis of *pmoB* and *pmoC* genes revealed similar topologies (Op den Camp et al. 2009). However, the placement of the *pmoCAB3* genes was often quite distant and sensitive to the particular sequence set and tree-building algorithm used. One phylogenetic conclusion at least is obvious: proteobacterial and verrucomicrobial *pmo* operons form distinct lineages without recent lateral gene transfer between the two groups. However, the relationship of the *pmoCAB3* genes to the other two operons in "*Methylacidiphilum*" is not clear.

A Bayesian phylogenetic analysis done by Tavormina et al. (2010) led to some interesting speculation about the deepest nodes of the trees. The topologies of

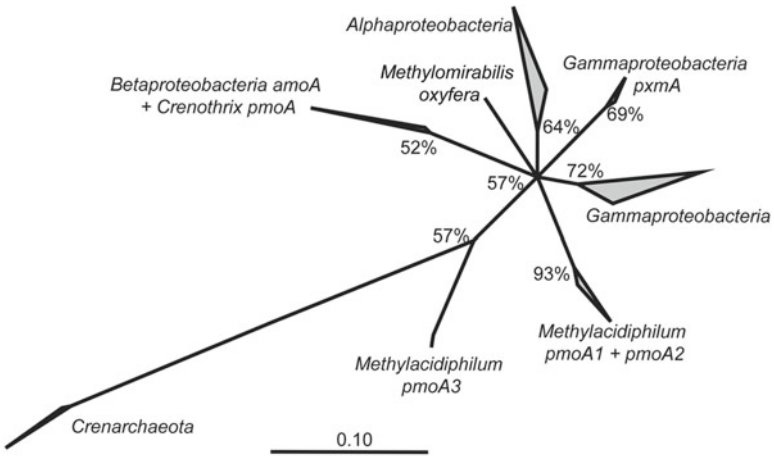

Fig. 9.4 Phylogenetic tree of partial (495 nucleotides) *pmoA/amoA* gene sequences of major groups of ammonia oxidizers and methanotrophs. The tree was constructed with TREE_PUZZLE, a quartet maximum-likelihood method, using a Schoeniger-von Hasseler distance calculation (Schmidt et al. 2002) and 50,000 iterations. Support values for the major nodes are given. The scale bar represents 0.1 changes per nucleotide position

amoA/pmoA and *amoB/pmoB* in their Bayesian trees support the hypothesis that "*Methylacidiphilum*" *pmoA3* genes are related to the *amoA* genes of ammonia-oxidizing *Crenarchaeota*. Nodes connecting verrucomicrobial *pmoA3/pmoB3* with *amoA/amoB* from *Crenarchaeota* were highly supported. Unfortunately, Bayesian phylogenies are notorious for delivering misleadingly high support values for deep nodes (e.g., Yang and Rannala 2005). In addition, the extremely deep branch to the *Crenarchaeota amoA* cluster may cause long-branch-attraction artifacts, a known hazard in any phylogenetic construction. Quartet-puzzling phylogenies constructed by Dunfield et al. (2007) and Stein et al. (2012) also include crenarchaeal *amoA*. These sometimes support a monophyletic *Verrucomicrobia* clade, but sometimes also support an affiliation of the verrucomicrobial *pmoA3* with crenarchaeal *amoA*. As is often the case, the conclusions are sensitive to different phylogenetic construction methods and datasets. A phylogeny constructed with a quartet-puzzling method and a modified dataset is shown in Fig. 9.4. The support for the *pmoA3* and crenarchaeal *amoA* node is present but weak at 57 %. Although the speculations of Tavormina et al. (2010) on the evolutionary connection of the *pmoA3* with *amoA* from *Crenarchaeota* are intriguing, they will require more data for confirmation.

Tavormina et al. (2010) also noted that the *pmoA1* and *pmoA2* genes in V4 also have a similar GC content as the overall genome (47 % vs. 45 %), but the *pmoA3* genes consist of only 37 % GC. They concluded that the *pmoA3* gene may be foreign to the genome. The Alien Hunter analysis noted earlier predicts the same, supporting the speculation of Tavormina et al. (2010) about the separate origin of the *pmoCAB3* operon compared to the other operons.

Conserved residues in derived PmoA and PmoC amino acid sequences of "*Methylacidiphilum*" were identical to those in the sequences from *Proteobacteria*. However, most of the conserved residues in proteobacterial PmoB were different in the *Verrucomicrobia*. These residues are involved in copper binding in proteobacterial pMMO, and their variability suggests a different metal coordination in the verrucomicrobial patterns (Op den Camp et al. 2009).

9.6 Pathways for C1 Metabolism and CO_2 Fixation

Aerobic methanotrophs first convert methane to methanol, which is oxidized to formaldehyde, formate, and CO_2. Methanotrophs employ a modular design for these metabolic steps (see Chistoserdova 2011) and Chap. 8 in this volume), with different species combining different modules for each step. The systems in "*Methylacidiphilum*" are not unique, although the particular combination of them is not seen in any known proteobacterial methanotroph. "*Methylacidiphilum*" has a gene encoding the large subunit (*mxaF*/*xoxF*) of a pyrroloquinoline quinone-dependent alcohol dehydrogenase of the methanol/ethanol family. No genes encoding the small subunit (*mxaI)* of methanol dehydrogenase or genes for several accessory proteins were found (Hou et al. 2008). Formaldehyde is probably oxidized via an enzymatic pathway using the C1-carrying cofactor tetrahydrofolate.

"*Methylacidiphilum*" does not have the methylene-H_4F dehydrogenase and methenyl-H4F cyclohydrolase enzymes that have been found in other methylotrophs, but does have a bifunctional *fol*D gene product that may perform these functions. A more common pathway in proteobacterial methanotrophs uses the C1-carrier tetrahydromethanopterin, but this is absent in "*Methylacidiphilum*." Formate is oxidized by a simple formate dehydrogenase.

Formaldehyde is a key intermediate in methane oxidation. It can be oxidized to CO_2 for energy generation or directly assimilated into cell material. Methanotrophs assimilate formaldehyde via either the serine cycle, in which formaldehyde and CO_2 are utilized in a one-to-one ratio to produce acetyl-CoA for biosynthesis, or via the RuMP pathway, in which formaldehyde is added to ribulose-5-monophosphate, which via sugar rearrangements produces glyceraldehyde-3-phosphate as a biosynthetic building block. Therefore, although there is some anapleurotic CO_2 assimilation, particularly in methanotrophs employing the serine cycle, these bacteria are considered heterotrophs that assimilate organic carbon. An estimated 85–95 % of biomass C in gammaproteobacterial methanotrophs and 50 % of the biomass C in alphaproteobacterial methanotrophs is derived from methane rather than CO_2 (Trotsenko and Murrell 2008).

Key enzymes of the RuMP pathway are absent in "*Methylacidiphilum*." Two key enzymes of the serine cycle, malyl-CoA lyase (*mcl*) and glycerate kinase (*glc*), could not be identified in the V4 genome, and other putative serine cycle enzymes were not arranged in genomic islands as they are in other methanotrophs, suggesting that their function may not be in the serine cycle. Hou et al. (2008) suggested that a modified serine cycle may operate, but further evidence would be required. The only clear pathway for C fixation identified in the genome of V4 was the Calvin-Benson-Bassham cycle.

RuBisCO, the key enzyme in the Calvin-Benson-Bassham (CBB) cycle, is present in both V4 and SolV. A number of methanotrophs possess the enzymes of the CBB, most in addition to other C1 assimilation pathways (Chistoserdova 2011). The CBB cycle is associated with a large energy use per mole of CO_2 fixed and was not considered a likely way to support growth on CH_4. However, Khadem et al. (2011) applied both $^{13}CH_4$ and $^{13}CO_2$ during growth experiments to verify the genome prediction that CO_2 is in fact the only carbon source for strain SolV. Genome and transcriptome analyses verified that all the genes necessary for the CBB cycle are present and expressed, most prominently the two genes encoding RuBisCO. Carboxysomes are thought to enhance the concentration of CO_2 for RuBisCO as it has a low affinity for CO_2, but strain SolV uses a non-carboxysome-associated form of RuBisCO, in agreement with the high concentrations of CO_2 required for growth (>0.3 %). The verrucomicrobial methanotrophs have been isolated from volcanic regions with high concentrations of CO_2, suggesting that there is no need to sequester CO_2 for growth (Khadem et al. 2011). Phylogenetic analysis of the *rbcL* gene suggests an LGT event from the *Actinobacteria*; however, this divergence must be ancient given that the *Verrucomicrobia rbcL* gene clade is clearly distinct from the *Actinobacteria*.

The autotrophic nature of the methanotrophic verrucomicrobia has recently been confirmed in strain V4 by Sharp et al. (2012) by performing a modified stable-isotope probing (SIP) technique labeling with both $^{13}CH_4$ and $^{13}CO_2$, individually and in combination. Strain V4 only assimilated $^{13}CO_2$, not $^{13}CH_4$, confirming the source of carbon for the verrucomicrobial methanotrophs is CO_2. In combination with quantitative PCR (qPCR) of verrucomicrobial-*pmoA* genes, this technique was then applied to geothermal soils from New Zealand, providing the first demonstration of the autotrophic nature of verrucomicrobial methanotrophs in situ. This study also demonstrated that there are several diverse clades of *Verrucomicrobia* methanotrophs present at the study site. Further application of the technique should allow an assessment of the overall diversity of *Verrucomicrobia* methanotrophs in nature.

9.7 Nitrogen Metabolism

The ability to fix gaseous nitrogen confers an advantage in environments where nitrogen is limiting. Genomic analyses of strains V4 and SolV suggest that these strains should be able to fix gaseous nitrogen, as they possess complete sets of genes for nitrogen fixation. Most of these genes have their organization in putative operons resembling those of *Methylococcus capsulatus* Bath (Ward et al. 2004), a proteobacterial methanotroph that has been shown to fix nitrogen. Khadem et al. (2010) demonstrated that the verrucomicrobial methanotroph strain SolV was able to fix nitrogen under low O_2 concentrations. Based on acetylene reduction assays and growth experiments, the nitrogenase enzyme of strain SolV appeared to be extremely O_2 sensitive compared to that of proteobacterial methanotrophs. Phylogenetic analysis of a concatenated set of the derived amino acid sequences of the *nifH*, *nifD,* and *nifK* genes showed that the verrucomicrobial nitrogenases group with those of the *Proteobacteria* and acidophilic *Leptospirillum* species that inhabit acid mine drainage. The tree is supportive of an LGT scenario with *Proteobacteria* (Khadem et al. 2010).

Ammonia is a strong competitive inhibitor of pMMO. Many methanotrophs oxidize ammonia to nitrite in two steps. In the first step, ammonia is oxidized to hydroxylamine by pMMO. In the second step, hydroxylamine is oxidized to nitrite. Many methanotrophs possess hydroxylamine cytochrome *c* oxidoreductase (HAO) activity to prevent accumulation of toxic hydroxylamine (Ward et al. 2004; Klotz et al. 2008). Recently, ammonia-responsive transcription of the *haoAB* genes encoding HAO have been reported for the methanotroph *Methylococcus capsulatus* Bath (Poret-Peterson et al. 2008). The finding of the *haoAB* gene on a plasmid in *Silicibacter pomeroyi* suggests a high mobility of these genes (Klotz et al. 2008). Both strains V4 and SolV of *Methylacidiphilum* possess an *haoA* gene encoding the large subunit of HAO. Phylogenetically, these groups with HAOs from methanotrophic *Proteobacteria* form a separate clade from the nitrifying bacteria *Nitrosococcus, Nitrosospira,* and *Nitrosomonas*. This may indicate lateral transfer of *haoA* from *Proteobacteria* to the *Verrucomicrobia* (Klotz et al. 2008), although in theory it may instead indicate transfer in the opposite direction.

The oxidation of hydroxylamine by HAO is accompanied by the production of small amounts of nitric oxide (NO). In "*Methylacidiphilum*," NO is then reduced to N_2O via a *norCB*-encoded nitric oxide reductase. This is found in some but not all proteobacterial methanotrophs (Stein and Klotz 2011). Phylogenetic analysis of *norC* and *norB* suggests that these are other strong candidates for lateral transfer. A small cluster of *norB* from *Aquificae, Thermus/Deinococcus,* and *Verrucomicrobia* cluster within a much larger clade of genes from *Proteobacteria* (Fig. 9.2). This phylogenetic clade may represent a thermotolerant form of the enzyme that was originally obtained via transfer from *Proteobacteria* and then shared among several thermophiles.

9.8 Conclusions

We are only beginning to understand the physiology of the "*Methylacidiphilum*"-type methanotrophs. They share many enzymatic modules with proteobacterial methanotrophs, but have a unique combination of these. Particularly special is their reliance on autotrophic C-assimilation, which seems counterintuitive for a bacterium growing on a reduced carbon substrate. Methanotrophs have often been defined in the literature as bacteria that can use methane as their sole energy and carbon source, but in fact "carbon" should be removed from this definition. Studies into the ecology and physiology of these bacteria are ongoing.

Acknowledgments C.E.S. is supported by fellowships from the Natural Sciences and Engineering Research Council of Canada (NSERC) and Alberta Innovates Technology Futures. The work was supported by an NSERC Discovery Grant (P.D.).

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