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Verrucomicrobial Methanotrophs

3

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3.1 Methane and Methanotrophy

The well-studied methanotrophic members of the Alpha- and Gammaproteobacteria have never been found in methane rich environments of extremely low pH. However, methane oxidation activity was detected in geothermal soils characterized by high temperatures (50–95 °C) and a pH as low as 1.0. From 2007 onward, the isolation of new aerobic acidophilic methane oxidizing bacteria from several acidic geothermal ecosystems was reported. They were all identified as new members of the Verrucomicrobia phylum and clustered in the genera *Methylacidiphilum* (thermophiles) and *Methylacidimicrobium* (mesophiles). This chapter reports on their discovery, biodiversity, genomics, metabolism, and biotechnological potential.

Methane is released to the atmosphere from natural and anthropogenic sources. The most important sources include natural ecosystems (e.g., wetlands, ruminants, and termites) and anthropogenic activities (e.g., rice paddy fields, landfills, and mining). In these systems, microbial biogenesis of methane is carried out by methanogenic Archaea during organic matter decay under anoxic conditions (Thauer 1998; Conrad 2009; Etiope et al. 2011). In addition, non-microbial methane is emitted to the atmosphere from geothermal regions like cold seeps, mud volcanoes, and fumaroles. This methane is produced primarily via the thermal decomposition of organic matter (>80 °C) in the earth's crust (Etiope and Klusman 2002; Conrad 2009; Etiope et al. 2011). Besides emission sources, sinks of methane are also present on our planet. Atmospheric methane can react with the hydroxyl radical OH leading to the formation of carbon dioxide and water vapor (Jacob 1999). Methane diffusing from anoxic production zones toward the atmosphere can be

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oxidized by both aerobic and anaerobic methane-oxidizing microorganisms, also known as methanotrophs (Forster et al. 2007; Conrad 2009). These microorganisms are assumed to be the major players in keeping the methane balance on our planet by oxidizing 50–80% of all methane produced before it can reach the atmosphere (Moss et al. 2000; Forster et al. 2007; Conrad 2009).

Microbial oxidation of methane can occur with or without molecular oxygen (O_2). To date we know that anaerobic oxidation of methane (AOM) can utilize various terminal electron acceptors: sulfate (Knittel and Boetius 2009; Milucka et al. 2012), nitrite (Ettwig et al. 2008), nitrate (Raghoebarsing et al. 2006; Haroon et al. 2013; Arshad et al. 2015), and manganese and iron oxides (Beal et al. 2009; Egger et al. 2015; Scheller et al. 2016). Many other microorganisms can oxidize methane aerobically, with O_2 as the terminal electron acceptor. For many years it was believed that aerobic methanotrophy was a phenotype only present in a few bacteria belonging to the phylum Proteobacteria, specifically in the classes Alphaproteobacteria (also named type II methanotrophs) and Gammaproteobacteria (type I methanotrophs) (Hanson and Hanson 1996; Dedysh et al. 2000; Op den Camp et al. 2009). The two groups differ in their pathways for biomass production, the ribulose monophosphate (RuMP) pathway in the Gammaproteobacteria versus the serine pathway in the Alphaproteobacteria (Hanson and Hanson 1996; Chistoserdova et al. 2009; Chistoserdova 2011). Other broad physiological and structural characteristics separating the two groups (cell shape, ultrastructure of intracytoplasmic membranes, the main phospholipid fatty acids, nitrogen fixation ability, presence of different monooxygenases, etc.) were also defined in the early years of research into these bacteria (Hanson and Hanson 1996), but recently many genera have been discovered that do not fit these sweeping generalizations (e.g., *Methylocella*, *Methylohalobius*, *Methylovulum*, and *Methyloceanibacter*; Dedysh et al. 2000; Heyer et al. 2005; Iguchi et al. 2011; Vekeman et al. 2016).

3.2 The Discovery of the Aerobic Verrucomicrobial Methanotrophs

The methanotrophic members of the Alpha- and Gammaproteobacteria have never been found in methane-rich environments of extremely low pH (Op den Camp et al. 2009). The most acidophilic proteobacterial methanotrophs known, species of *Methylocella* and *Methylocapsa*, grow in peat environments at pHs as low as 4.2 (Dedysh et al. 1998, 2002; Dunfield and Dedysh 2010). However, methane oxidation activity was detected in geothermal soils in the Solfatara at Pozzuoli near Naples (Italy), a site characterized by high temperatures (50–95 °C) and a pH as low as 1.0 (Castaldi and Tedesco 2005). The hydrogen sulfide present in the hot fumarolic gas at this site is oxidized into sulfuric acid biotically or abiotically, thereby forming an extremely acidic ecosystem. Biogeochemical evidence for methane oxidation at extremely low pH was confirmed in late 2007 to early 2008 by three independent studies that obtained isolates of new aerobic methane-oxidizing bacteria from several acidic volcanic samples from the Solfatara; from Hell's Gate, Tikitere (New Zealand); and from the Uzon Caldera, Kamchatka (Russia) (Pol et al. 2007;

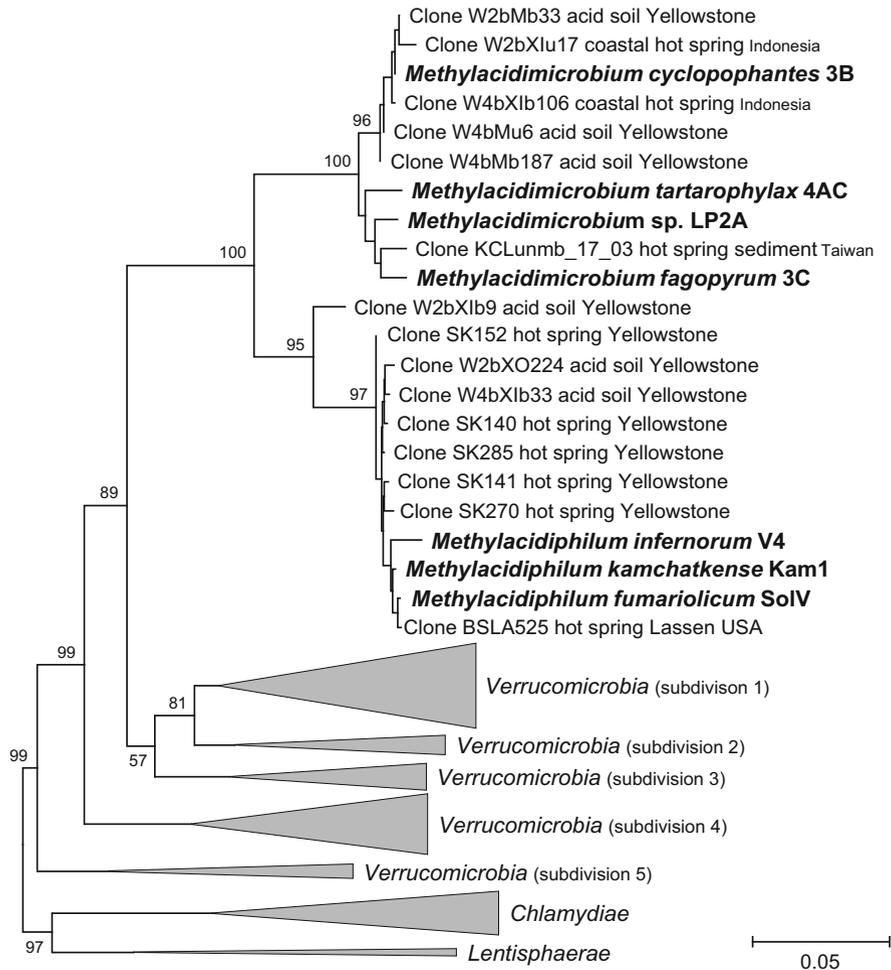


Fig. 3.1 Phylogenetic tree of 16S rRNA gene sequences of methanotrophic and other Verrucomicrobia showing the evolutionary relationships of the methanotrophic mesoacidophilic *Methylacidimicrobium* strains (3B, 3C, LP2A, and 4AC), the methanotrophic thermoacidophilic *Methylacidiphilum* strains (V4, SolV, and Kam1), and other members of the phylum Verrucomicrobia and other selected phyla. Cultivated representatives are indicated in boldface. Chlamydiae and Lentisphaerae species were used as outgroup

Dunfield et al. 2007; Islam et al. 2008). All three isolates were able to grow at pH 1 and temperatures up to 65 °C (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Op den Camp et al. 2009). Interestingly, based on 16S ribosomal RNA gene analysis, all three strains (SolV, V4, and Kam1) were identified as members of the Verrucomicrobia phylum and phylogenetically formed a single genus-level cluster for which the name *Methylacidiphilum* was suggested (Fig. 3.1) (Op den Camp et al. 2009). This genus has yet to be taxonomically validated because of difficulties in maintaining a viable culture in reference culture collections, but is a useful label for

consistent identification. The link between a member of the widely distributed Verrucomicrobia phylum and a major global process like the methane cycle was an exciting discovery. Despite the importance of Verrucomicrobia in many different ecosystems, which is suggested by their abundance in 16S rRNA amplicons from environmental DNA extracts, the portion of cultivated representatives within the Verrucomicrobia is quite low (Hugenholtz et al. 1998). 16S rRNA gene phylogenetic analyses have allowed a division of the phylum into five subdivisions (Fig. 3.1), but the physiology of the different subdivisions is still poorly understood (Wagner and Horn 2006).

3.3 Diversity of Verrucomicrobia methanotrophs

After the discovery of the thermoacidophilic genus *Methylacidiphilum*, van Teeseling et al. (2014) and Sharp et al. (2014) isolated, characterized, and described four new species of mesophilic acidophilic verrucomicrobial methanotrophs from low-temperature geothermal sites: the soil of the Solfatara crater, which is at the center of the Campi Flegrei caldera, near Naples (Italy), and a geothermally impacted soil in Reporoa, New Zealand. The 16S rRNA genes of these new isolates were very similar to each other, but were less than 90% identical to those of the *Methylacidiphilum* species described earlier. Furthermore, PmoA- and MxaF-/XoxF-based phylogenies showed similar clustering compared to the 16S rRNA-based phylogeny (Keltjens et al. 2014; van Teeseling et al. 2014). The new genus name *Methylacidimicrobium* was proposed for these mesophilic verrucomicrobial methanotrophs, including the species *Methylacidimicrobium tartarophylax* 4AC, *Methylacidimicrobium fagopyrum* 3C, *Methylacidimicrobium cyclopophantes* 3B, and *Methylacidimicrobium* sp. LP2A (van Teeseling et al. 2014). Again these names are not yet taxonomically validated.

Together, the thermophilic and mesophilic methanotrophs described form a coherent class-level clade within the Verrucomicrobia phylum (Fig. 3.1). 16S rRNA gene sequences belonging to this group have since been detected in other acidic geothermal habitats of Europe, Asia, and North America, indicating their widespread occurrence in such systems (Kozubal et al. 2012; Li et al. 2016; Gagliano et al. 2014). In an extensive survey of environments in Canada and New Zealand using high-throughput sequencing of 16S rRNA amplicons, Sharp et al. (2014) found putative methanotrophic Verrucomicrobia in samples covering a broad temperature range (22.5–81.6 °C), but only under acidic conditions (pH <5.0) and only in geothermal systems. They could not be detected in acidic non-geothermal methane-rich environments like fens and bogs, suggesting that factors other than pH alone also affect their distribution. However, similar 16S rRNA sequences have recently been detected in highly acidic microbially induced concrete corrosion zones of sulfide-impacted sewage pipes (Pagaling et al. 2014). This suggests that methanotrophic Verrucomicrobia are indeed present in some non-geothermal ecosystems, at least H₂S-rich ones. The factors controlling the ecological range of these bacteria are not yet completely understood, but may be related to their rare earth metal requirement (see below) in addition to their preference for highly acidic conditions.

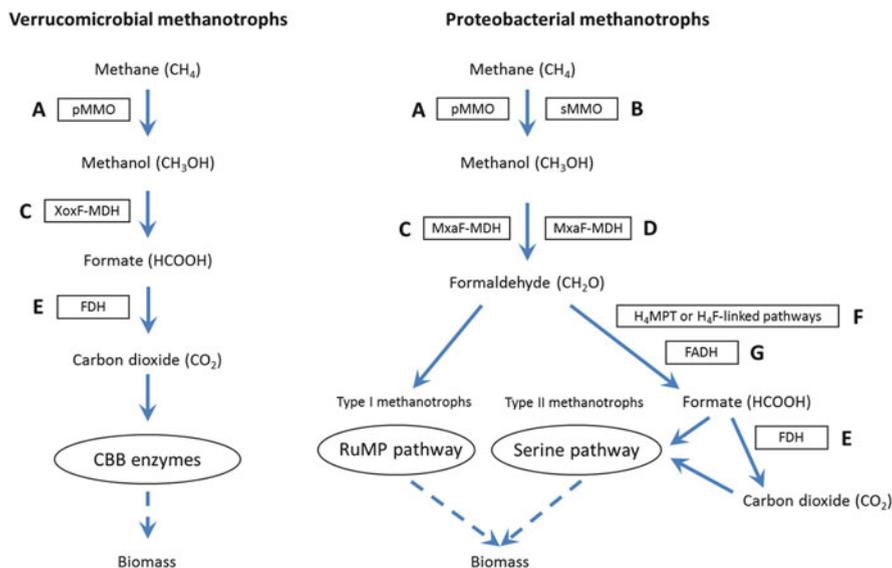


Fig. 3.2 Methane oxidation pathways in verrucomicrobial (left) and proteobacterial (right) methanotrophs with all enzymes (boxes) involved. **(A)** Particulate methane monooxygenase (pMMO); **(B)** soluble methane monooxygenase (sMMO). **(C)** Lanthanide-dependent methanol dehydrogenase (XoxF-MDH); **(D)** calcium-dependent methanol dehydrogenase (MxaF-MDH); **(E)** formate dehydrogenase (FDH); **(F)** multienzyme cofactor-linked C1 transfer pathways (H_4MPT = tetrahydromethanopterin, H_4F = tetrahydrofolate); **(G)** formaldehyde dehydrogenase (FADH). Formaldehyde is assimilated in type I proteobacterial methanotrophs via the ribulose monophosphate (RuMP), and formate is the main branch point to the serine pathways in type II proteobacterial methanotrophs. In verrucomicrobial methanotrophs, carbon dioxide is fixed using the Calvin-Benson-Bassham (CBB) cycle enzymes

3.4 Genomics and Metabolism of Verrucomicrobia Methanotrophs

The aerobic oxidation of methane has the net reaction, $\text{CH}_4 + 2\text{O}_2 \rightarrow \text{CO}_2 + 2\text{H}_2\text{O}$ ($\Delta G^\circ = -773$ KJ per mol CH_4), but proceeds microbially via the intermediates methanol (CH_3OH), formaldehyde (CH_2O), and formate (CHOOH) (Chistoserdova et al. 2009; Hanson and Hanson 1996) (Fig. 3.2). In this reaction scheme, electrons from methane are transported to a membrane-bound electron transport chain using a pyrroloquinoline quinone cofactor to cytochrome *c* (methanol dehydrogenase) or NAD (in formaldehyde oxidation systems and formate dehydrogenase). The terminal electron acceptor in aerobic methane oxidation is O_2 .

The first step in methane oxidation is catalyzed by the methane monooxygenase enzyme (MMO) converting methane into methanol. This enzyme exists in two distinct forms; the soluble cytoplasmic form (sMMO, NADH-dependent) and the particulate membrane-associated form (pMMO, cytochrome *c* dependent) (Hanson and Hanson 1996; Fig. 3.2). In general, sMMO is located in the cytoplasm, and pMMO is situated in

the intracytoplasmic membranes (ICMs) present in many proteobacterial methanotrophs (Nguyen et al. 1998; Brantner et al. 2002). The soluble cytoplasmic methane monoxygenase (sMMO) is only present in a limited number of methanotrophs, usually in addition to pMMO (Chistoserdova 2011), but occasionally as the sole MMO (*Methylocella*, *Methyloferula*, and *Methyloceanibacter*) has only the sMMO enzyme (Dedysh et al. 2005; Dunfield and Dedysh 2010; Vekeman et al. 2016). The completed circular genomes of *M. inferorum* strain V4 and *M. fumariolicum* strain SolV lack the genes encoding for sMMO subunits (Pol et al. 2007; Hou et al. 2008; Op den Camp et al. 2009; Anvar et al. 2014). Draft genomes of *M. kamchatkense* (Kam1) and the mesophilic *Methylacidimicrobium* strains also lack sMMO-encoding genes (Erikstad and Birkeland 2015; Sharp et al. 2014; van Teeseling et al. 2014). On the other hand, complete *pmoCAB* operons encoding pMMO are found in the genome of every strain. The *Methylacidimicrobium* strains either contain a single *pmoCAB* operon (van Teeseling et al. 2014) or in the case of strain LP2A two nearly identical operons and an orphan third *pmoC* copy (Sharp et al. 2014). The completed thermoacidophilic *Methylacidiphilum* genomes each contain three complete *pmoCAB* operons and an orphan fourth *pmoC* copy (Op den Camp et al. 2009), while the draft genome of *M. kamchatkense* strain Kam1 showed three complete *pmoCAB* operons and a fourth operon without the *pmoB* gene (Op den Camp et al. 2009; Erikstad et al. 2012). Remarkably, the three paralogous operons in *Methylacidiphilum* are phylogenetically divergent from one another by up to 50% amino acid sequence. Alien Hunter, a program that uses nucleotide k-mer frequencies to identify potential lateral gene transfer (Vernikos and Parkhill 2006), predicts that the *pmoCAB3* operon, the most divergent of the three, has been obtained by lateral gene transfer from another organism (Sharp et al. 2013). However, as no other organism is known with a similar operon, the source of this transfer is unknown.

This presence of diverse *pmo* copies in the *Methylacidiphilum* genomes is not unique, as some proteobacterial methanotrophs also possess multiple, divergent copies of *pmo* operons. A divergent *pmoCAB2* was reported to be present in many type II proteobacterial methanotrophs, including the model strain *Methylocystis* SC2 (Tchawa Yimiga et al. 2003). Baani and Liesack (2008) reported that the enzymes encoded by the two paralogous operons in this model strain showed different apparent K_m values. Recently, a sequence-divergent *pmo* (named *pxm*) was also found in some type I proteobacterial methanotrophs (Tavormina et al. 2011). The existence of sequence-divergent copies may indicate different physiological functions under different environmental conditions. In *Methylacidiphilum* species, the functions of the three paralogous operons are not yet clear, although initial expression studies have been performed. Khadem et al. (2012a) showed that *pmoCAB1* and *pmoCAB2* operons of strain SolV are highly but differentially expressed under oxygen limitation and oxygen excess, respectively. Interestingly, the third *pmoCAB* operon (*pmoCAB3*) of strain SolV was not expressed under any of conditions tested (Khadem et al. 2012a). In *M. kamchatkense* strain Kam1, growth on methanol instead of methane resulted in a downregulation of all *pmoA* genes (Erikstad et al. 2012).

The second enzyme involved in the aerobic oxidation of methane is the PQQ-dependent methanol dehydrogenase (MDH) converting methanol into formaldehyde (Fig. 3.2). This enzyme includes a large and a small subunit encoded by *mxoFI* genes and needs pyrroloquinoline quinone as cofactor and a cytochrome *c* electron acceptor encoded by the *mxoG* gene. Remarkably, in all available verrucomicrobial aerobic methanotrophic strains (both *Methylacidiphilum* and *Methylacidimicrobium* spp.), the well-studied *mxoFJGIRSACKLDEHB* cluster encoding the canonical *mxoF*-type MDH was absent and substituted by *xoxFGJ*, while biogenesis of the cofactor pyrroloquinoline quinone is encoded by *pqqABCDEF* (Hou et al. 2008; Op den Camp et al. 2009; Pol et al. 2014; Keltjens et al. 2014; van Teeseling et al. 2014; Sharp et al. 2014). After the observation that growth of *M. fumariolicum* SolV was strictly depended on the addition of mudpot water from its natural environment, it was discovered that the purified XoxG-type MDH of *M. fumariolicum* strain SolV contains lanthanides at the catalytic site, while previously studied *mxoF*-type MDHs have calcium as active metal at this site (Pol et al. 2014; Keltjens et al. 2014). It was also shown that the growth of strain SolV is dependent on lanthanides at submicromolar concentrations, which could replace the mudpot water.

The presence of a lanthanide in the PQQ catalytic center seems to make XoxF-type MDHs more efficient catalysts in methanol conversion, since they efficiently oxidize not only methanol but also formaldehyde. The oxidation of methanol into formate may have major implications for the diversity of methyl- and methanotrophic catabolism and anabolism. The field of methano- and methylotrophy is a rapidly expanding puzzle of redundant anabolic and catabolic possibilities and opportunities (Chistoserdova et al. 2009; Chistoserdova 2011).

The third step in the proteobacterial aerobic oxidation of methane is the conversion of formaldehyde to formate. Formaldehyde is a toxic intermediate and must be maintained at nontoxic levels (Chistoserdova 2011). Different formaldehyde-oxidizing systems are present in methylotrophs (Chistoserdova 2011). Formaldehyde oxidation can be carried out by a formaldehyde dehydrogenase (FADH; Fig. 3.2). This single enzyme is linked to NAD or mycothiol (Chistoserdova 2011). In addition, formaldehyde oxidation can be performed by multienzyme cofactor-linked C1 transfer pathways. Two important pathways for formaldehyde oxidation in methylotrophs require H₄MPT (tetrahydromethanopterin) or H₄F (tetrahydrofolate) as cofactors (Chistoserdova et al. 2009; Chistoserdova 2011). The H₄MPT pathway is common in proteobacterial methanotrophs but appears to be missing from the genomes of all the methanotrophic Verrucomicrobia. Some parts of a H₄F pathway are present, but this pathway has not been verified. *M. fumariolicum* strain SolV lacks FADH as well as H₄MPT pathway-encoding genes, but the lanthanide-dependent XoxF-type MDH was shown to oxidize methanol directly to formate (see above), so a specialized formaldehyde oxidation system may not be needed by these methanotrophs.

The last step in methane oxidation is the conversion of formate into carbon dioxide by formate dehydrogenase (FDH; Fig. 3.2). The complete genome of strains V4 and SolV and the draft genome of strain Kam1 show that formate oxidation is

probably conducted by a NAD-dependent formate dehydrogenase and a membrane-bound formate dehydrogenase (Pol et al. 2007; Hou et al. 2008; Anvar et al. 2014; Erikstad and Birkeland 2015). In proteobacterial methanotrophs, formaldehyde and/or formate is assimilated via two main pathways: the *RuMP* and serine pathways which are important for Gammaproteobacteria (type I) and Alphaproteobacteria (type II) methanotrophs, respectively (Hanson and Hanson 1996; Chistoserdova et al. 2009). In the *RuMP* pathway, the unique enzymes hexulose-6-phosphate synthase and hexulose-6-phosphate isomerase catalyze the reactions to assimilate formaldehyde to form glyceraldehyde-3-phosphate as an intermediate (Hanson and Hanson 1996). In the serine pathway, the exclusive reactions are catalyzed by serine hydroxymethyltransferase (STHM), hydroxypyruvate reductase (HPR), malate thiokinase (MTK), and malyl coenzyme A lyase (MCL) (Hanson and Hanson 1996). In this pathway, both formate and carbon dioxide are utilized to produce acetyl coenzyme A for biosynthesis (Crowther et al. 2008; Šmejkalová et al. 2010). Based on the full genomes of the verrucomicrobial methanotrophs strain SolV and V4, and the transcriptome data of strain SolV, it is clear that these bacteria do not follow either the *RuMP* or the serine pathway (Hou et al. 2008; Op den Camp et al. 2009; Anvar et al. 2014). Rather, the verrucomicrobial methanotrophs are autotrophs, fixing carbon from carbon dioxide using the Calvin-Benson-Bassham (CBB) cycle (Khadem et al. 2011; Sharp et al. 2012). Recently, Rasigraf et al. (2014) also showed autotrophic carbon dioxide fixation via the Calvin-Benson-Bassham cycle by the denitrifying methanotroph *Methylomirabilis oxyfera* belonging to the NC10 phylum.

In addition to methane oxidation, *M. fumariolicum* strain SolV is able to fix N_2 at low oxygen concentrations using an extremely oxygen-sensitive nitrogenase (Khadem et al. 2010). Furthermore, the storage of carbon in strain SolV was demonstrated. Glycogen was formed in the cells once ammonium was depleted and methane still present (Khadem et al. 2012b). Gammaproteobacterial methanotrophs may also produce glycogen, while alphaproteobacterial methanotrophs have been shown to produce PHB (polyhydroxybutyric acid) as a storage compound (Linton and Cripps 1978; Pieja et al. 2011a, b; Eshinimaev et al. 2002).

3.5 Knallgas Bacteria

Recently, it was shown that the methanotroph *Methylacidiphilum fumariolicum* SolV could also grow as a real “Knallgas” bacterium consuming hydrogen/carbon dioxide in the absence of methane (Mohammadi et al. 2017). Two sets of hydrogen uptake hydrogenase genes were identified in the genome of strain SolV, encoding an oxygen-insensitive (*hhy*-type) and an oxygen-sensitive (*hup*-type) enzyme. Transcriptome analysis revealed that the *hhy*-type hydrogenase was constitutively expressed and active. This hydrogenase was supposed to support growth on hydrogen only at oxygen concentrations below 1.5%. When oxygen was further reduced to

below 0.2%, expression of the oxygen-sensitive *hup*-type hydrogenase was induced, and the growth rate increased to about 60% of the rate on methane.

In an independent study, Carere et al. (2017) showed that the environmental isolate *Methylacidiphilum* sp. RTK17.1 sustained aerobic respiration and carbon fixation using methane and hydrogen as electron donors either in concert or separately depending on substrate availability. This lifestyle may have facilitated expansion of their niche space in geothermally influenced surface soils.

Taken together, these results suggest that in their natural environments, where both hydrogen and methane might be limiting, verrucomicrobial methanotrophs may operate primarily as “Knallgas” bacteria or prefer a mixotrophic lifestyle. In view of these findings, the role of hydrogen in methanotrophic ecosystems has to be revised, especially in soil, and related to consumption of atmospheric methane.

3.6 Biotechnology

The low cost of natural gas and biogas compared to other fuels makes them promising feedstocks for bioconversions. Possible bioproducts include polymers, single-cell protein, vitamins, carotenoids, compatible solutes, lipids, or methanol (Strong et al. 2015). As of yet, there are no published reports of the use of verrucomicrobial methanotrophs for bioconversion or indeed for any other biotechnological use. At the moment one can only speculate that their unique biochemistry compared to all other methanotrophs may present unique possibilities for bioproduct discovery and production.

Ecologically, the Verrucomicrobia methanotrophs occupy a niche separate from proteobacterial methanotrophs and may therefore also present unique possibilities for environmental biotechnology. For example, biofiltration of methane is a useful way of reducing the carbon footprint of waste gas streams that are economically not feasible to recover because they are intermittent, remote, and contaminated or have low flow rates or low methane contents (Hettiarachchi and Hettiaratchi 2011). Via biofiltration, the potent greenhouse gas methane is oxidized to CO₂, a gas with a global warming potential 34 times less than methane. The presence of H₂S in biogas or natural gas (“sour gas”) is a general problem for the sustained operation of a methane biofilter and in some situations is also problematic for the economic recovery of fuel methane. The Verrucomicrobia methanotrophs are naturally adapted to sour gas sources and may therefore be useful in biofiltration of sour gas. Their unique tolerance may even make sour gas streams viable feedstocks for biotechnology without a desulfurization step. The observed co-occurrence of sulfur- and methane-oxidizing acidophiles in soured pipes (Pagaling et al. 2014) indicates the possibility for simultaneous H₂S and CH₄ removal from waste gas.

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