Minireview

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Unexpected metabolic versatility among type II methanotrophs in the Alphaproteobacteria

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Abstract: Aerobic methane-oxidizing bacteria, or methanotrophs, play a crucial role in the global methane cycle. Their methane oxidation activity in various environmental settings has a great mitigation effect on global climate change. Alphaproteobacterial methanotrophs were among the first to be taxonomically characterized, nowadays unified in the *Methylocystaceae* and *Beijerinckiaceae* families. Originally thought to have an obligate growth requirement for methane and related one-carbon compounds as a source of carbon and energy, it was later shown that various alphaproteobacterial methanotrophs are facultative, able to grow on multi-carbon compounds such as acetate. Most recently, we expanded our knowledge of the metabolic versatility of alphaproteobacterial methanotrophs. We showed that *Methylocystis* sp. strain SC2 has the capacity for mixotrophic growth on H₂ and CH₄. This mini-review will summarize the change in perception from the long-held paradigm of obligate methanotrophy to today's recognition of alphaproteobacterial methanotrophs as having both facultative and mixotrophic capabilities.

Keywords: acetate; hydrogen; hydrogenase; methanotrophs; pMMO; sMMO.

Introduction

Methane (CH₄) is the most abundant organic compound in the Earth's atmosphere, and it is the second most important

greenhouse gas in the atmosphere with a 20-year global warming potential (GWP₂₀) 84 times greater than CO₂ (Allen 2016). With its current atmospheric concentration of 1.87 ppmv, CH₄ contributes approx. 15% to the total greenhouse effect (Saunio et al. 2016). Therefore, the methane cycle is one of the most important biogeochemical processes in the Earth's system. Starting from the industrial era, but in particular over the past 15 years, the atmospheric concentration of methane has significantly increased, thereby leading to an imbalance in the global methane budget (Allen 2016; Saunio et al. 2016). This budget is defined by the methane sources and sinks on which the aerobic methane-oxidizing bacteria, or methanotrophs, have a major impact.

Methanotrophs are a subset of a physiological group known as methylotrophs, microorganisms that grow on one-carbon compounds such as methanol and methylated amines. A common characteristic of all aerobic methanotrophs is their ability to oxidize methane to carbon dioxide and water with the formation of methanol, formaldehyde, and formate as intermediates. Methanol, formed from methane, is subsequently oxidized to formaldehyde by the methanol dehydrogenase located in the periplasmic space of the cell. Most of the reducing equivalents for energy conservation and growth are formed by the oxidation of formaldehyde to formate and finally to carbon dioxide. Alternatively, formaldehyde may be assimilated into cell carbon or used as a precursor for synthesis of key intermediates of the central carbon metabolism (Dedysh and Knief 2018; Bodelier et al. 2019).

Methanotrophic bacteria are found in three phyla: *Proteobacteria*, *Verrucomicrobia*, and candidate phylum NC10. While the first methanotroph was isolated early last century (Söngen 1907), it needed additional 60 years until Whittenbury et al. (1970) characterized over 100 methanotroph isolates that later were shown to belong to the *Proteobacteria*. This pioneering work, but also their ubiquitous distribution, made proteobacterial methanotrophs to the most well-studied methane oxidizers. They will be discussed in greater detail below. Another 40 years had to pass before the known diversity of methane-oxidizing bacteria was expanded beyond *Proteobacteria*. Methanotrophs in the phylum *Verrucomicrobia* are specialized on living in

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extremely acidic (pH 1.8–5.0) geothermal (up to 82 °C) sites, as deduced from their habitat preferences. Members of the candidate phylum NC10 or, more specifically, cells of ‘*Candidatus Methyloirabilis oxyfera*’ carry out a nitrite-dependent anaerobic oxidation of methane by the intracellular formation of oxygen through dismutation of two NO molecules to N₂ and O₂. The intracellularly formed oxygen is used for oxidizing methane along the pathway known for conventional aerobic methanotrophs. Thus, “*Ca. Methyloirabilis oxyfera*” is more likely a cryptic aerobe rather than a strict anaerobe, albeit residing in anoxic environments. Unlike proteobacterial methanotrophs, which prefer to assimilate carbon from reduced forms of C₁ carbon, *Verrucomicrobia* and NC10 methanotrophs are autotrophs that use methane only as energy source. These bacteria fix carbon from carbon dioxide using the Calvin–Benson–Bassham (CBB) cycle (Khadem et al. 2011; Rasigraf et al. 2014). For more detailed information, we refer to (Op den Camp et al. 2018) for *Verrucomicrobia* methanotrophs and to (Ettwig et al. 2010; Versantvoort et al. 2018) for “*Ca. Methyloirabilis oxyfera*”.

Proteobacterial methanotrophs act as a natural biofilter in diverse methanogenic environments, such as peat bogs, rice paddies, freshwater sediments, and landfills. Inhabiting the oxic-anoxic interfaces, methanotrophs reoxidize 10 to 90% of the methane produced by methanogenic archaea before its emission to the atmosphere. Proteobacterial methanotrophs form phylogenetically distinct groups within the *Gammaproteobacteria* (type Ia, type Ib, and type Ic) and *Alphaproteobacteria* (type IIa and type IIb) (Knief 2015). Type I and type IIa methanotrophs have been historically differentiated by a set of characteristic features (Hanson and Hanson 1996) of which, apart from phylogenetic affiliation, only a very few are still considered valid (Knief 2015). In principle, type I methanotrophs use the ribulose monophosphate pathway (RuMP) for the assimilation of carbon into cell biomass. Their cells contain intracytoplasmic membranes (ICMs) organized in the form of stacks of flattened vesicles that fill most of the cytoplasmic space and are oriented perpendicular to the cell membrane. In contrast, type IIa methanotrophs of the genera *Methylocystis* and *Methylosinus* (*Methylocystaceae*) use the serine pathway for carbon assimilation. They possess the ethylmalonyl-coenzyme A (EMC) pathway for glyoxylate regeneration and an assimilatory network through which a substantial fraction of methane-oxidation derived CO₂ is incorporated back into the serine/EMC pathway (Yang et al. 2013). The ICMs of their cells are stacks of flattened vesicles oriented parallel to the outer membrane and located around the cell perimeter. *Methylocystis* and *Methylosinus* are among the most oligotrophic methanotrophs and therefore widely distributed in upland soils where these bacteria may

contribute to atmospheric methane oxidation (Figure 1). In fact, high-affinity methanotrophs act as a biological sink for 28–38 Tg per year of atmospheric methane (Allen 2016; Saunio et al. 2016). The moderately acidophilic type IIb methanotrophs of the family *Beijerinckiaceae* differ from type IIa methanotrophs in a number of cellular characteristics. For example, cells of *Methylocella* and *Methyloferula* do not possess ICMs, while in *Methylocapsa*, the ICMs are always located on one side of the cell (Dedysh et al. 2000; Vorobev et al. 2011). Furthermore, some members of the family *Beijerinckiaceae* contain, in addition to the serine pathway, the complete set of genes for the CBB cycle, though the functional role remains unclear (Khmelenina et al. 2018). Since the first detailed methanotroph characterization in 1970, it was a long-held paradigm that proteobacterial methanotrophs are defined by their obligate use of methane and related one-carbon compounds as sole source of carbon and energy. Research over the last two decades, however, has revealed that alphaproteobacterial methanotrophs are metabolically more versatile and have the ability for facultative growth (Figure 1). Most recently, we showed that these bacteria are also capable of growing mixotrophically on H₂ and CH₄ (Figure 2) (Hakobyan et al. 2020). The following sections will be devoted to how our perception on alphaproteobacterial methane oxidizers changed from being obligate methanotrophs towards having capabilities to grow facultatively and mixotrophically.

Biochemistry of obligate methane oxidation

The methane monooxygenase (MMO) enzyme, which is only produced by methanotrophic bacteria, catalyzes the oxidation of methane to methanol. This enzyme exists in two forms, a particulate form (pMMO) and a soluble form (sMMO). Both evolutionarily distinct forms of MMO require an input of two electrons and two protons for the methane oxidation step. For sMMO, the possible source of electrons is NADH/H⁺ (Semrau et al. 2010). In *Gammaproteobacteria* methanotrophs, pMMO activity is directly coupled to the oxidation of methanol to formaldehyde (Kalyuzhnaya et al. 2015). The mechanism is different in *Alphaproteobacteria* methanotrophs. Here, the electrons for pMMO activity are supplied through the ubiquinone pool after the oxidation of NADH by complex I (Bordel et al. 2019).

The pMMO, a copper-containing enzyme, is housed in the ICMs of methanotrophic bacteria, while sMMO is a cytoplasmic non-heme iron enzyme complex (Ross et al.

Figure 1: Obligate and facultative methanotrophy among cultured alphaproteobacterial methanotrophs. In flooded soils, methanotrophs usually inhabit the oxic-anoxic interfaces, but individual methanotrophic populations may prefer different CH₄/O₂ mixing ratios for optimal growth activity. Methane production through the activity of methanogenic archaea occurs in the deeper soil layers. In addition to *Methylocystis* and *Methylosinus* (pMMO), members of the genera *Methylocella* (sMMO) and *Methylocapsa* (pMMO) are known to thrive in moderately acidic environments such as peat bogs. *Methylocystis*, *Methylosinus*, and *Methylocapsa* are also widely distributed in well-aerated upland soils where they may contribute to the high-affinity oxidation of atmospheric CH₄ (1.87 ppm), in addition to largely uncultured methanotroph groups termed “upland soil cluster alpha and gamma” (USCα, USCγ) and some conventional gammaproteobacterial methanotrophs such as *Methylocaldum*. While *Methylocella* prefers acetate over CH₄ as growth substrate, the utilization of multi-carbon compounds by the other three genera, i.e., *Methylocystis*, *Methylosinus*, and *Methylocapsa*, is presumably a survival strategy to maintain under fluctuating (flooded soils) or limiting (upland soils) CH₄ conditions.

2017). Except *Methylocella* and *Methyloferula*, all known aerobic methanotrophs possess pMMO. This enzyme has a fairly narrow substrate specificity, oxidizing alkanes and alkenes with a length of no more than five carbon atoms. It consists of three subunits (PmoB, PmoA, PmoC) organized as an $\alpha_3\beta_3\gamma_3$ trimer (Lieberman et al. 2005). The active site is proposed to be located in the di-copper center of the PmoB subunit, as deduced from crystallographically modeled pMMO protein (Ross et al. 2017). Genes encoding pMMO are

organized in the *pmoCAB* operon, which is usually located on chromosomal DNA. Previous studies have shown that the production of pMMO is accompanied by enhanced expression of genes involved in the biosynthesis of membrane lipids. Their co-expression with *pmoCAB* ensures adequate formation of ICMs for the integration of pMMO subunits (Kao et al. 2004). More recently, we have shown that the membrane fraction of logarithmically growing cells of *Methylocystis* sp. strain SC2 accounts for 23% of its total proteome, with 15% of that fraction being the pMMO protein (Hakobyan et al. 2018). In *Escherichia coli*, the membrane fraction only comprises 8% of its proteome.

Most proteobacterial methanotrophs possess two nearly sequence-identical copies of the *pmoCAB1* operon and, in addition, single copies of the *pmoC* gene (Knief 2015). In addition to the two copies of *pmoCAB1*, many *Methylocystaceae* members were shown to possess a third pMMO-encoding gene cluster, termed *pmoCAB2* (Yimga et al. 2003). Enzymes encoded by *pmoCAB1* (pMMO1) and *pmoCAB2* (pMMO2) differ in their affinity for CH₄ (Figure 1). In methanotrophs that contain both pMMO isozymes, the production of pMMO1 takes place at elevated methane concentrations, while pMMO2 is capable of oxidizing even atmospheric CH₄ (Baani and Liesack 2008). Because the ammonia monooxygenase of nitrifying bacteria is a homolog of pMMO, ammonia acts as a cometabolic substrate for the pMMO enzyme. In particular, the high-affinity methane oxidation by pMMO2 is negatively affected by increasing ammonia concentrations. Mostly expressed at a significantly lower level than pMMO1, ammonia acts as a competitive inhibitor and blocks the accessibility of the active site of pMMO2 for methane (Dam et al. 2014; Hakobyan et al. 2018).

The sMMO enzyme is present in various representatives of type II methanotrophs as well as in *Methylococcus capsulatus* and some other strains of type I methanotrophs. It catalyzes the pyridine nucleotide-dependent oxidation of methane by molecular oxygen to methanol and has, contrary to pMMO, a broad substrate range. In addition to methane, sMMO can catalyze the oxidation of alkanes, alkenes, aromatic hydrocarbons, and other multi-carbon substrates. The sMMO enzyme is a three-component complex consisting of a hydroxylase, a reductase, and a regulatory protein B. The non-heme iron-containing hydroxylase contains the hydrophobic active-site cavity. It contains a bridged di-iron center, where CH₄ and O₂ interact to form methanol (Rosenzweig et al. 1993; George et al. 1996). The active hydroxylase complex is an $\alpha_2\beta_2\gamma_2$ dimer, encoded by *mmoX*, *mmoY*, and *mmoZ*. Reductase and regulatory proteins are encoded by *mmoB* and *mmoC*, respectively. The genes encoding sMMO are arranged in the following order: *mmoRGXYBZDC* (Murrell et al. 2000; Semrau et al. 2010).

Various *Methylocystaceae* members are able to produce both enzyme systems – pMMO and sMMO. In methanotrophs that produce both forms of MMO, the “copper switch mechanism” plays a key role in regulating the reciprocal expression of pMMO and sMMO (Murrell et al. 2000; Semrau et al. 2010), meaning that both the transition from sMMO to pMMO production and the concurrent increase in cellular ICM content is induced by increasing copper concentrations. More recent studies discovered a unique copper-binding compound called methanobactin, which is produced in many methanotrophs in response to low copper conditions (DiSpirito et al. 2016). Methanobactin was shown to be a small (<1300 Da) ribosomally synthesized post-translationally modified peptide acting as a chalkophore with high affinity for copper binding (DiSpirito et al. 2016).

The second step of the methane oxidation pathway is catalyzed by methanol dehydrogenase. Many methanotrophs and various methylotrophs, but also some gram-negative non-methylotrophic bacteria, contain two different enzymes that convert methanol to formaldehyde; namely MDH and XoxF. MDH is a pyrroloquinoline quinone (PQQ)-dependent enzyme, comprised of two large catalytic subunits (MxaF) and two small subunits (MxaI). It binds calcium as a cofactor that assists PQQ in catalysis. During methanol oxidation, PQQ is reduced to the corresponding quinol (PQQH₂) followed by a two-step transfer of electrons to the terminal oxidase (Khmelenina et al. 2018). XoxF exhibits approximately 50% amino acid sequence identity to MxaF. The XoxF protein is a homodimer consisting of two large subunits. Instead of calcium, it requires light lanthanides, such as La³⁺ and Ce³⁺, for its catalytic activity. In comparison to MDH, XoxF has a higher affinity to methanol with an affinity constant as low as 0.8 μM. Lanthanides regulate the reciprocal switch between *mx*A and *xox*F expression, with the complete inhibition of *mx*A expression already at micromolar lanthanide concentrations.

Facultative methanotrophy

Proteobacterial methanotrophs were initially thought to rely on methane as their sole source of carbon and energy, thus being “obligate methanotrophs”. Some of these methanotrophs can also utilize the intermediates of methane oxidation, such as methanol, formate, and formaldehyde and, in addition, methylamines. However, in the first decade of this century, it was shown that in addition to one-carbon compounds, particular methanotrophs are able to utilize multi-carbon compounds for growth, therefore being facultative methanotrophs. The existence of such facultative methanotrophs had been discussed for a long

time (reviewed in Semrau et al. 2011; Theisen et al. 2005) until the pioneering research on *Methylocella* provided the first validated evidence for facultative methanotrophy (Figure 1) (Dedysh et al. 2005). *Methylocella* is an unusual methanotroph with respect to both its cell architecture and its preference for carbon sources (Crombie et al. 2014). The cells only possess sMMO and thus lack ICMs and, in addition, prefer to grow on multi-carbon compounds rather than methane (acetate, pyruvate, succinate, malate, and ethanol) (Dedysh et al. 2005). *Methylocella* strains grow on acetate more efficiently than on methane (Figure 1). In fact, sMMO promoter expression is repressed in the presence of acetate (Smirnova et al. 2018).

Later, facultative methanotrophy was also shown for *Methylocystis* and *Methylocapsa* (Figure 1) (Belova et al. 2011; Dunfield et al. 2010; Im et al. 2011). Unlike *Methylocella*, members of the latter two genera possess pMMO and, in consequence, a conventional cell architecture (ICMs). The paradigm of *Methylocystis* being an obligate methanotroph was disproved first for *Methylocystis bryophila* strain H2s^T and *Methylocystis* sp. strain SB2. While both strains showed a clear preference for growth on methane, they also were able to grow slowly on acetate or ethanol in the absence of methane (Belova et al. 2011; Im et al. 2011; Vorobev et al. 2014). In H2s^T cells grown for several transfers on acetate, the ICMs were maintained, albeit in reduced form, and mRNA transcripts of pMMO were detectable. These H2s^T cells resumed their growth on methane faster than those kept for the same period of time without any substrate. No growth occurred on other multi-carbon compounds (Belova et al. 2013).

Early experiments with ¹⁴C-labeled acetate had already revealed restricted incorporation of acetate-derived carbon into lipids and amino acids (Eccleston and Kelly 1973), indicating that methanotrophs can produce acetyl-CoA and channel it into biosynthetic pathways. Furthermore, the dynamic incorporation of acetate into poly-β-hydroxybutyrate by a type II methanotroph was demonstrated using ¹³C NMR spectroscopy (Vecharskaya et al. 2001). More recently, *Methylocystis hirsuta* was shown by genome-scale metabolic modeling (GSSM) and experimental approaches to have the ability for growth on acetate (Bordel et al. 2019). The metabolic model predicts that these bacteria use the glyoxylate assimilation cycle, within the EMC pathway, to transform acetyl-CoA to glycerate. Transcriptomic analysis of *Methylocystis* sp. strain SB2 revealed that, when grown on ethanol, the alcohol is converted to acetyl-CoA which is channeled into the EMC pathway (Vorobev et al. 2014). Nonetheless, all growth experiments on acetate or ethanol yielded significantly less biomass than those with methane, thereby confirming

methane as the preferred growth substrate for members of the family *Methylocystaceae*.

Mixotrophy

In addition to facultative methanotrophy, *M. bryophila* strain H2s^T was shown to conduct mixotrophy in the presence of two substrates, methane and acetate (Belova et al. 2011). Indeed, the utilization of acetate as a supplementary carbon source during growth on methane or methanol has already been proposed long time ago (Eccleston et al. 1973; Patel et al. 1977). In case of *M. bryophila* strain H2s^T, the growth rate was slower during mixotrophic growth on methane and acetate than with methane as the only substrate for growth, but biomass yield was higher. Furthermore, acetate was consumed much faster in the presence than in the absence of methane (Belova et al. 2011).

Recently, several physiological and ecological studies have demonstrated that hydrogen is an ubiquitous and easily accessible energy source for a wide range of soil microorganisms (Piche-Choquette and Constant 2019). Hydrogen is continuously produced on our planet by abiogenic and biogenic processes, particularly by biochemical processes such as fermentation, nitrogen fixation, and photosynthesis. Under anoxic conditions, H₂ can be produced in various environments including rice paddies, peat soils, swamps, inland water-logged (ponds,

lakes, rivers) and marine muds, sewage slugde, horse manure, human feces, and elsewhere in nature (Morita 1999). The production of H₂ is not restricted to anaerobic environments. It also can be produced in aerated soils, with N₂ fixation being the main biological process responsible for H₂ production under micro-oxic conditions (Piche-Choquette and Constant 2019).

Atmospheric hydrogen, which can diffuse into surface soils, has a mixing ratio as low as 0.53 ppmv. However, N₂-fixing legume nodules may act as hot spots for hydrogen, with concentrations ranging between 9000 and 27,000 ppmv (Khdhiri et al. 2017; Piche-Choquette et al. 2018). Diffusive H₂ fluxes at the soil-nodule interface are able to stimulate both low-affinity (K_m ca. 1000 ppmv) and high-affinity (K_m ca. 100 ppmv) H₂ oxidation (Piche-Choquette et al. 2018). The use of H₂ as an alternative energy source may help microorganisms to cope with environmental fluctuations in available carbon and energy sources (Greening et al. 2016; Piche-Choquette and Constant 2019).

Aerobic hydrogen oxidation by hydrogenases involves the transfer of electrons into the electron transport chain, finally resulting in ATP generation. Hydrogenases are metalloenzymes classified by the metal content in the active site into [Fe]-hydrogenases, [FeFe]-hydrogenases, and [NiFe]-hydrogenases. The most widespread hydrogenases in aerobic soil bacteria are [NiFe]-hydrogenases (Vignais et al. 2007). The group of [NiFe]-hydrogenases is quite heterogeneous and divided into five subgroups,

Figure 2: Mixotrophy in *Methylocystis* sp. strain SC2. Cells of strain SC2 were incubated with and without the addition of 2% H₂ under 6% CH₄/3% O₂ atmosphere. In the control treatment, CH₄ is used as the sole source of carbon and energy. During mixotrophic growth, strain SC2 combines hydrogen respiration with CH₄-based carbon assimilation. Hydrogen is oxidized by Group 1d uptake hydrogenase, leading to a significantly increased ATP level relative to the control. In consequence, the H₂ treatment doubles the biomass production, with less CH₄ consumed. In addition, H₂ oxidation provides electrons required for CH₄ oxidation by pMMO, while these are supplied through NADH oxidation by complex I when grown without H₂. In both H₂ and control treatments, reducing equivalents and ATP may also be generated through

fermentation-based methanotrophy, with acetate being the main product. However, the amount of acetate produced and excreted into the medium was markedly less with H₂ than without H₂ as electron donor.

having either membrane-bound or cytosolic localization (Piche-Choquette and Constant 2019). The majority of [NiFe]-hydrogenases are O_2 tolerant and have relatively low catalytic activity.

Hydrogen-oxidizing bacteria active in the soil-nodule interface primarily belong to the *Proteobacteria*, *Actinobacteria*, and *Acidobacteria* (Khdhiri et al. 2017). In the proximity of H_2 high-concentration sources (e.g., legume nodules), the rates of atmospheric CH_4 oxidation in farmland and poplar soils were shown to be decreased by 67 and 78%, respectively (Piche-Choquette et al. 2018). The strong negative correlation between low-affinity H_2 oxidation and high-affinity CH_4 oxidation led to the conclusion that these two processes be performed by the same microbes. Indeed, various methanotrophs have been shown to contain genes encoding different types of [NiFe]-hydrogenases responsible for hydrogen oxidation (Carere et al. 2017; Greening et al. 2016). Thus, there is an increasing body of evidence indicating that methanotrophic bacteria have a more versatile metabolic potential than originally thought, including different mixotrophic lifestyles relying on alternative carbon and energy sources (Piche-Choquette et al. 2018).

Already many years ago, hydrogenase activities in proteobacterial methanotrophs have been shown to contribute reducing power for methane oxidation (Hanczar et al. 2002), recycle endogenous hydrogen produced during nitrogen fixation (Chen et al. 1987) and drive the non-productive oxidation of chlorinated solvents in *Methylosinus trichosporium* OB3b (Shah et al. 1995). This prompted us to explore hydrogen utilization by our model organism, *Methylocystis* sp. strain SC2 (Hakobyan et al. 2020).

Strain SC2 has a high genetic potential to utilize H_2 as an alternative energy source for aerobic respiration. Its genome encodes four different [NiFe]-hydrogenases: Group 1d, Group 1h/5, Group 2b, and Group 3b. The ability of strain SC2 to utilize hydrogen was assessed in growth experiments with and without 2% H_2 under different mixing ratios of CH_4 and O_2 . In CH_4 - and O_2 -limiting conditions (6% CH_4 /3% O_2), hydrogen oxidation significantly enhanced the biomass yield of strain SC2 with about 50% less CH_4 consumed. H_2 addition did not have any significant effect on the growth rate and biomass yield of strain SC2 under elevated CH_4 and O_2 starting concentrations (Figure 2).

The experiments further showed that long-term incubation (37 days) in fed-batch mode with 2% H_2 under CH_4 - and O_2 -limiting conditions had a positive effect on the growth of strain SC2. Proteomic analysis of samples taken from these long-term incubations revealed that adding 2% H_2 to the cultures induced the expression of two

hydrogenases, Group 1d and Group 2b (Figure 2). Group 1d is a low-affinity membrane-bound uptake hydrogenase responsible for electron transfer to the electron transport chain, while Group 2b is a regulatory hydrogenase that forms a complex with a histidine protein kinase. It is thought that this hydrogenase rapidly recognizes H_2 in the environment and transmits the signal to a response regulator, which in turn controls transcription of the hydrogenase genes (Vignais et al. 2007; Greening et al. 2016). The concurrent expression of these two hydrogenases led not only to the oxidation of H_2 and, in consequence, a significantly increased cellular ATP level, but also to an almost complete redirection of CH_4 from energy generation to the assimilation into cell carbon (Figure 2). This more than doubled the increase in cell biomass relative to the control without H_2 addition. Moreover, in the presence of hydrogen, the expression of NADH dehydrogenase (complex I), whose activity is responsible for the supply of electrons required for CH_4 oxidation by pMMO in type II methanotrophs (Bordel et al. 2019), was significantly repressed. Presumably, under H_2 availability, the functional role of complex I was taken over by Group 1d uptake hydrogenase (Hakobyan et al. 2020).

Concluding remarks

Aerobic methanotrophs play a major role in mitigating global climate change. Over the last two decades, hundreds of studies have therefore been conducted to detect and analyze methanotrophic communities directly in the environment using *pmoA* as a marker gene for methanotroph detection. Because the *pmoA* gene sequence is highly conserved among methanotrophs, well-designed real-time quantitative PCR (qPCR) and reverse transcription qPCR (RT-qPCR) assays allow for the analysis of methanotrophic diversity, abundance, and activity in any given environment. The environmental studies showed that *Methylocystis* species are amongst the most ubiquitously distributed methanotrophs and able to survive in environments where methane is limited, or even close to the atmospheric level. Our knowledge gained over the last decade about the metabolic versatility of *Methylocystis* species thus provides the underlying basis for understanding their survival under adverse conditions. The ability of various *Methylocystaceae* members to produce two pMMO isozymes with different substrate affinities may be one evolutionary adaptation to fluctuating methane availability (Figure 1). Another adaptation is the ability of *Methylocystis* species to slowly grow on acetate in the absence of methane. In fact, stable isotope probing with

^{13}C -labeled acetate under aerobic conditions resulted in a labeling of uncultured *Methylocystis* species in rice field soil, demonstrating that the labeled carbon was metabolized and incorporated into cell biomass (Leng et al. 2015). Given the wide distribution of [NiFe]-hydrogenases among *Methylocystaceae* members, the proven ability of strain SC2 for mixotrophic growth on H_2 and CH_4 broadens the known metabolic versatility of *Methylocystis* species to cope with changing nutrient availability (Figure 2). Strain SC2 has been shown to oxidize H_2 from elevated starting concentrations (2%) to below the detection limit of the H_2 sensor (0.02%) in batch cultivation mode. Thus, H_2 -based mixotrophy may be relevant not only in the proximity of legume nodules in farmland soils, but also in the oxic-anoxic interfaces of methanogenic environments where methanotrophs encounter greater variations in methane, hydrogen, and oxygen concentrations (Piche-Choquette and Constant 2019; Piche-Choquette et al. 2018).

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