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Current Opinion in Microbiology

Physiology of atmospheric methane-oxidizing bacteria

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The biological sink for atmospheric methane consists of atmospheric methane-oxidizing bacteria (atmMOB) that persistently oxidize atmospheric methane as carbon and energy source and conventional methanotrophs that transiently oxidize atmospheric methane after exposure to elevated methane concentrations. The ecology and environmental activity of atmMOB have been studied for several decades, but until the first detailed characterization in 2019 of an atmMOB in pure culture that can grow with air as the sole energy (methane, carbon monoxide and molecular hydrogen) and carbon (methane and carbon dioxide) source, their physiology was mostly unexplored. Here we summarize the available knowledge about atmMOB physiology, including the kinetics of atmospheric methane oxidation, energy yields during growth on methane and other trace gases from air, carbon assimilation and physiological diversity. We use this background to identify knowledge gaps that should be targeted to support future research on atmMOB.

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Current Opinion in Microbiology 2025, **88**:102656

This review comes from a themed issue on **Environmental Microbiology**

Edited by **Chris Greening** and **Cornelia Welte**

Available online xxxx

https://doi.org/10.1016/j.mib.2025.102656

Atmospheric methane oxidizing bacteria

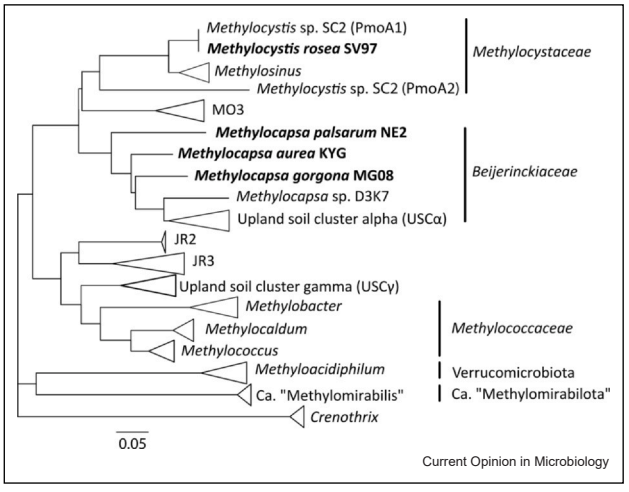
Biological atmospheric methane (CH_4) oxidation is an important component of the global CH_4 cycle, primarily associated with soils and trees. Measurements of atmospheric CH_4 uptake by upland soils and trees indicate a combined sink strength of up to 100 Tg CH_4 yr^{-1}, which is more than 10% of the atmospheric CH_4 sink [1,2]. The process is attributed primarily to atmospheric methane-oxidizing bacteria (atmMOB), which we define as methanotrophs capable of persistently oxidizing atmospheric CH_4 (approximately 2 ppm). This distinguishes them from methanotrophs unable to oxidize atmospheric CH_4 and those that may transiently oxidize atmospheric CH_4 but ultimately depend on elevated CH_4 ($> > 2$ ppm) to sustain their metabolic activity, often called flush-feeders; however, the contribution of flush-feeders to the size of the atmospheric CH_4 sink, relative to that of atmMOB, remains unknown.

Much of our understanding of atmMOB is derived from *in situ* activity measurements combined with the detection of the *pmoA* gene, which encodes a subunit of the particulate methane monooxygenase (pMMO) enzyme. The pMMO enzyme catalyzes the oxidation of CH_4 to methanol, the initial step of aerobic methanotrophy, in a reaction that requires both O_2 and electrons. The *pmoA* gene serves as a phylogenetic marker for methanotrophs, and soils exhibiting atmospheric CH_4 uptake were found to be dominated by *pmoA* clades that clustered separately from known methanotroph genera with cultivated representatives [3]. This supported the consensus that atmMOB are specialized CH_4 oxidizers, physiologically, phylogenetically, and ecologically distinct from canonical methanotrophs [4]. AtmMOB in soils exposed to low CH_4 concentrations were further indicated to have a high affinity for CH_4, seemingly distinct from the kinetic properties reflected by CH_4 uptake rates in soils exposed to higher CH_4 concentrations [5].

Most analyses of *pmoA* diversity in soils exhibiting atmospheric CH_4 uptake identify either upland soil cluster alpha (USCα) or upland soil cluster gamma (USCγ) [6–8]. USCα is affiliated with the family *Beijerinckiaceae* within Alphaproteobacteria and characteristic of neutral to acidic upland soils [6]. USCγ belongs to the Gammaproteobacteria and is associated with neutral to alkaline soils [6]. In addition to USCα and USCγ, clades within the families *Methylocystaceae*, *Beijerinckaceae*, *Methylococcaceae* and *Crenotrichaceae* have been found in environments with net atmospheric CH_4 uptake [4,6,7,9–15] and may represent hitherto uncultivated atmMOB. The identity of the methanotrophs responsible for CH_4 uptake associated with upland trees is still unresolved.

Aside from some reports of sustained atmospheric CH_4 consumption by *Methylocystis* [16–18], *Methylocapsa gorgona* MG08, which is closely related to the USCα, was the first confirmed atmMOB in pure culture, and the first

Figure 1



Neighbor-Joining tree of PmoA (protein) sequences showing confirmed atmMOB (in bold). The tree was constructed using MEGA11 and contains only a few representative clades for reference and should not be considered a comprehensive set of PmoA groups or a phylogenetic analysis. For a comprehensive analysis of *pmoA* diversity, see Ref. [3].

for which growth on trace gases (CH_4, H_2 and CO) and other gases (CO_2) from air as sole carbon and energy sources was demonstrated [19,20]. The cultivation techniques that permitted isolation of *M. gorgona* MG08 also confirmed growth on air by the previously isolated methanotrophs *Methylocapsa palsarum* NE2, *Methylocapsa aurea* KYG and *Methylocystis rosea* SV97 [20], implying that the ability to persistently oxidize atmospheric CH_4 is not restricted to methanotrophs within the USCα and USCγ (Figure 1). A common feature of these atmMOB strains is the presence of the membrane-bound pMMO enzyme, while the cytoplasmic soluble methane monooxygenase (sMMO) has consistently been absent in all strains confirmed as atmMOB to date.

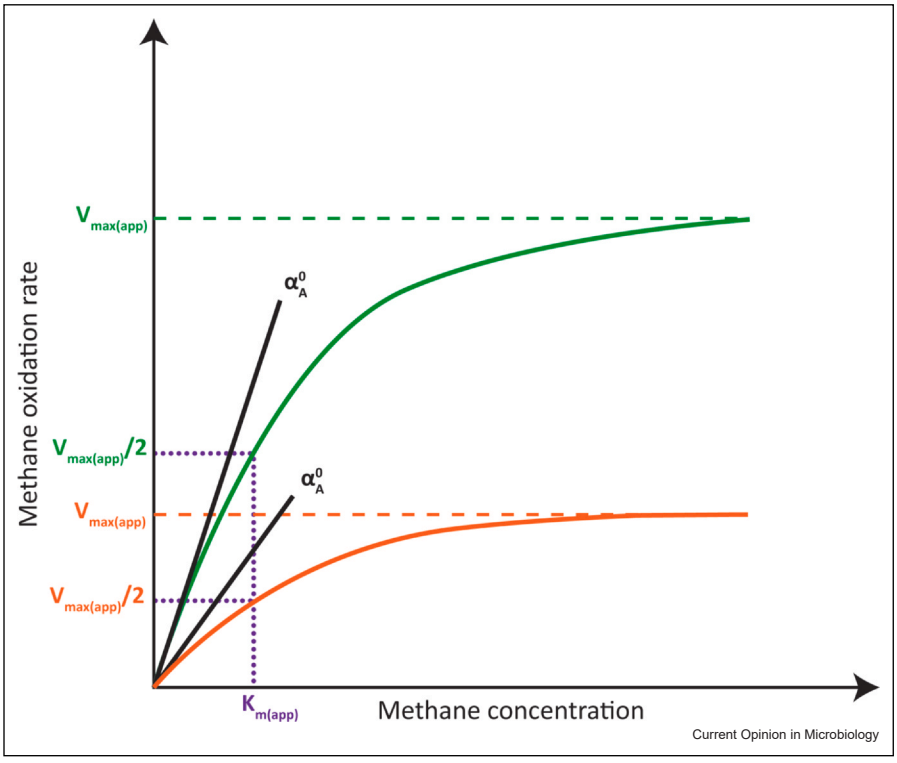
Atmospheric CH_4 oxidation

The first indication of biological atmospheric CH_4 oxidation was reported in dry swamps in 1982 [21], while the first evidence for CH_4 assimilation in methanotrophs at atmospheric CH_4 concentrations was published in 2000 [22]. Over the years, atmospheric CH_4 uptake was observed in forest soils [4,23], tropical soils [24], grasslands and meadows [25], landfill cover soils [26], deserts [27], heathlands [28], dryland rice soils [29,30], tundra soils [31], caves [32] and surfaces of birch, spruce [33] and mosses [34]. In 1992, it was reported that atmospheric CH_4 uptake in soils is characterized by a low apparent Michaelis kinetic constant ($K_{m(app)}$) [5], 'apparent' meaning that the conditions of the enzyme reaction are not fully known [35]. The low $K_{m(app)}$ indicated that the microorganisms responsible for atmospheric CH_4 uptake may have a higher affinity for CH_4 than other methanotrophs [5]. However, as later discussed by Dunfield, the high affinity hypothesis is problematic because simple Michaelis–Menten kinetics might not be applicable to MMOs because these enzymes require three reactants (CH_4, O_2 and reductant such as reduced cytochromes) [35]. The inappropriateness of $K_{m(app)}$ as a measure of enzymatic affinity for CH_4 is illustrated by the observation that changes in CH_4 concentration alter the $K_{m(app)}$ of *Methylocystis* strain LR1 [16]. However, this observation does not exclude the possibility that there is a difference in enzymatic affinity for CH_4 between methanotrophs that can live on atmospheric CH_4 and those that cannot. To address this, it is essential to obtain cell-free and stable pMMO enzymes from atmMOB for activity assays [36] and further identify the structural basis for differences in affinity between atmMOB and other methanotrophs. However, while the structural properties and active site of atmMOB pMMO can be studied with current methodology, and recent advances to reconstitute the pMMO into bicells have been shown to recover the activity, the activity of reconstituted pMMOs seems to remain lower than that of cellular pMMOs, suggesting that characterizations of atmMOB pMMO kinetics must await further methodological development [37,38].

In addition to being an imprecise measure of substrate affinity, the $K_{m(app)}$ also fails to provide information about the rate of substrate uptake at atmospheric CH_4 concentrations. The apparent specific affinity (a_s^0) was suggested as a better way to describe the ability to collect nutrients under limitation [39] than substrate affinity. In terms of methanotrophy, the specific affinity indicates the rate at which low concentrations of CH_4 can be oxidized by a cell [16,35], representing the pseudo-first-order rate constant of CH_4 oxidation at low concentrations (Figure 2). The specific affinity arises from the combination of the $K_{m(app)}$ and the maximal obtainable velocity of the reaction ($V_{max(app)}$) and is expressed as $a_s^0 = \frac{V_{max(app)}}{K_{m(app)}}$ [35]. Thus, a high specific affinity, or in other words, a high CH_4 oxidation rate at low CH_4 concentration, can be obtained by both lowering the $K_{m(app)}$ and increasing the $V_{max(app)}$ [35].

Two strains that were shown to grow on air, *M. gorgona* MG08 and *M. palsarum* NE2, have the two highest specific affinities for CH_4 so far demonstrated: 1.01×10^{-9} L cell^{-1} h^{-1} and 3.30×10^{-9} L cell^{-1} h^{-1}, respectively [20]. A combination of proteomics and kinetics data indicates that these two strains obtain their high specific affinities in two different ways. While *M. gorgona* MG08 has a lower $K_{m(app)}$ (48.53 nM), within the range observed for CH_4 uptake in upland soils [5], *M. palsarum* NE2 has a much higher $K_{m(app)}$ (412 nM), a higher $V_{max(app)}$, and a higher a_s^0. The higher $V_{max(app)}$ and a_s^0 in *M. palsarum* NE2 are likely driven by higher cellular

Figure 2



Specific affinity as a parameter for evaluating the ability to consume atmospheric CH_4. The figure shows methane oxidation rates at different methane concentrations for two hypothetical methanotrophs, in red and green. These curves demonstrate how two organisms with the same $K_{m(app)}$ can have very different methane oxidation rates at low methane concentrations, and thus that a low $K_{m(app)}$ is not a good criterion for the ability to oxidize atmospheric CH_4 fast enough to survive or grow. Methane oxidation rates at low concentrations are illustrated by the specific affinity (a^0_s), which represents the pseudo-first-order rate constant at low methane concentrations and is calculated as $V_{max(app)}$ divided by $K_{m(app)}$. This means that atmMOB can obtain high methane oxidation rates at low methane concentrations by both having a low $K_{m(app)}$ and a high $V_{max(app)}$. Figure is modified from Ref. [40].

pMMO content during growth at atmospheric CH_4 concentrations, relative to *M. gorgona* MG08 [20], but protein quantification is required to test this hypothesis. The two strains were cultivated on filter membranes floating on water during these kinetics experiments, meaning that they were not exposed to limitations from the rate of CH_4 diffusion in water, but only the solubility of CH_4 in the thin water film that surrounds the cells. See the work of Schmider and colleagues for examples of how to perform these kinetics experiments and calculate the CH_4 concentrations in the water surrounding the cells [20]. It is important to note that despite being provided the same concentrations in the surrounding air, active cells in larger volumes of water might have lower concentrations of CH_4 available than those growing on surfaces surrounded by a thin water film due to diffusion limitations.

M. gorgona MG08 and *M. palsarum* NE2 grow at both high (1000 ppm) and atmospheric CH_4 concentrations. Comparative proteomics revealed that they express the same pMMO genes at both concentrations [20]. A different physiology, which may allow growth at both low (indications for growth at 10 ppm) and high (> 600 ppm) CH_4 concentrations, was observed in *Methylocystis* strain SC2. This strain carries two *pmoCAB* operons with different CH_4 $K_{m(app)}$ and a^0_s of their pMMO products, pMMO1 and pMMO2, respectively. At low CH_4 concentration, the SC2 cells upregulated expression of pMMO2, resulting in a $K_{m(app)}$ of 111 nM, similar to *M. gorgona* MG08. At higher CH_4 concentrations, pMMO1 upregulation and a $K_{m(app)}$ of 9200 nM were observed [17]. It should be noted that the experiments with *Methylocystis* SC2 were done in liquid culture containing NH_4^+ as a nitrogen source. The NH_4^+ could have interfered with the estimation of $K_{m(app)}$ for CH_4, as indicated by the influence of CH_4 on the $K_{m(app)}$ of ammonia oxidation [41].

In some methanotrophs, possibly including members of *Methylocystis* and *Methylosarcina*, uptake of atmospheric CH_4 concentrations was observed for a limited time after stimulation by higher CH_4 concentrations [18,30]. While this lifestyle, called flush-feeding [35], falls outside our

definition of atmMOB, they might represent an important contribution to the biological sink for atmospheric CH_4.

Energy yield, maintenance and growth

The CH_4 uptake rate determines how much energy is available from CH_4 per unit of time to support maintenance and growth at an atmospheric CH_4 concentration. Based on estimates of the temperature dependence of average microbial maintenance energies (Aerobic and Anaerobic), the value of 2.8 kJ per C-mol biomass per hour at 20°C (4.5 kJ at 25°C) [42] became a benchmark energy yield for evaluating whether a given CH_4 oxidation rate could support methanotrophic maintenance [15,18,43,44]. Maintenance energy is the minimum energy required for a cell to stay alive, and can be defined by the costs associated with activities such as osmoregulation and turnover of macromolecular molecules [45]. The assumption is that energy yields above that minimum would allow growth. Around 0.5 kJ $Cmol^{-1}$ h^{-1} (0.38–0.71 kJ $Cmol^{-1}$ h^{-1}) at 20°C was recently shown to support methanotrophic growth on CH_4, CO and H_2 from air by *M. gorgona* MG08 and other methanotrophic species [20]. While 0.5 kJ $Cmol^{-1}$ h^{-1} to support growth seems low compared to the earlier benchmark value of 2.8 kJ $Cmol^{-1}$ h^{-1} to cover maintenance requirements, life is expected to be supported by much lower energy yields in oxic marine sediments. Here, a median of 2.23×10^{-18} Watts are available per cell [46], as opposed to 1.1×10^{-15} Watts per cell for atmMOB growing on air in pure culture [20] (energy yields for atmMOB converted from kJ per cell to Watts per cell without normalization to C-mol to match environmental data). The reasons why such low energy yields can support life in subseabed sediments are likely to be low maintenance requirements and low mortality rates, which may allow generation times as long as thousands of years [47,48]. Essentially, the less energy is required for repairs, and the lower the mortality, the less energy is required for a cell to stay alive and to grow fast enough to maintain the population size. This is also why the energy yields under close to optimal conditions in atmMOB pure culture are not sufficient information for evaluating whether growth can be sustained in nature. We must also consider the abiotic and biotic factors that influence mortality (e.g. viral load and predation) [49] and maintenance energy requirements (e.g. radiation or oxygen radicals that can cause protein and RNA damage [50]).

In 1999, Conrad theorized that 7.2×10^7 atmMOB cells can be energetically sustained in a gram of dry soil when provided with atmospheric CH_4 concentration, and assuming a maintenance requirement of 2.8 kJ $Cmol^{-1}$ h^{-1} at 20°C [43]. Considering the recent empirical evidence for atmMOB growth at the more than five times lower energy yield of ~0.5 kJ $Cmol^{-1}$ h^{-1}, 7.2×10^7 cells is likely to be a more than fivefold underestimation of a theoretically sustainable atmMOB population size under ideal environmental conditions and with constant access to atmospheric CH_4. However, environmental quantitative polymerase chain reaction estimates indicate $0.3{\times}10^8$ to $1.2{\times}10^8$ *pmoA* genes gDW^{-1} [15], which is in the range of the population size originally theorized by Conrad. Thus, since atmMOB seem to require less energy than previously assumed, but do not reach larger population sizes, atmMOB in the environment are likely to be constrained beyond the limitations of the atmospheric CH_4 concentration, possibly by CH_4 diffusion limitations and variables such as the above-mentioned viral infection, predation or physical and chemical cellular damage.

To understand the environmental control of atmMOB, it might be necessary to consider the physiological differences between atmMOB lineages: Due to differences in the molecular machinery, maintenance and growth requirements may vary considerably, even between species within the same genus. For example, with a higher number of ATP synthase c-ring subunits, less energy per proton, and thus a smaller transmembrane concentration gradient potential is required for ATP production, possibly favoring growth at low substrate concentrations, but at the cost of growth efficiency [47]. Also, the size, volume, and permeability of cells matter because these variables influence the cost of maintaining ion homeostasis and plasmolysis (contracted cytoplasm) that is considered the major cell maintenance cost during starvation [51].

Temperature is an example of an environmental variable that may influence the maximal population size and activity of atmMOB, but where differences between individual species may occur due to differences in temperature adaptation. Temperature influences soil moisture content and the solubility and diffusion of gases, and therefore CH_4 availability to cells [52] and atmMOB CH_4 uptake [35,53]. However, temperature also controls how much energy is required to support cellular maintenance [42,54], and how cells distribute their resources between protein biosynthesis and other cell functions [55]. Correspondingly, it has been shown that temperature controls how much CH_4 is required to support cell division in methanotrophs [56].

One of the most fundamental properties of atmMOB that should be studied to understand their environmental dynamics and physiology is growth. AtmMOB are assumed to grow very slowly. This is partly based on their extremely slow recovery time after disturbance, for example, in agriculture, which can be in the range of decades [57]. It is also based on a more general assumption that atmospheric CH_4 concentrations are low and limited by the solubility of CH_4 in water and diffusion constraints in soil, and therefore very restrictive for growth rates and population sizes [53,57]. However,

to obtain useful predictions of atmMOB responses to climate change and identify ways to optimize their growth in agricultural soils or other systems, we need to move beyond these generalizations and learn to precisely measure atmMOB growth rates.

Still, no published growth rates of atmMOB at atmospheric CH_4 concentrations exist, in pure culture or in soil or other environments, and studies of how environmental variables influence atmMOB physiology are lacking. Estimations of general bacterial growth rates in upland soils indicate that most populations grow slowly, with relative rates averaging below 0.05 day^{-1} [58,59]. Microbial populations in nature may predominantly be in states that resemble stationary or near-stationary growth, where cell division primarily replaces dying cells and only small increases or decreases in the population size, if any, occur over time [60]. Furthermore, only some of the cells within a population may be growing at any given time. Heterogeneity in cellular functions within populations is a potential key aspect of slow growth at low substrate concentrations. For example, heterogeneity was observed for *Saccharomyces cerevisiae*, *E. coli* and *B. subtilis* [60], with only subsets of slow-growing populations retaining the ability to grow under a given condition. This heterogeneity, which may be triggered stochastically in genetically identical populations, may contribute to increasing the chances that a subset of the population can enter exponential growth when conditions change [61].

Alternative energy sources and carbon assimilation

In addition to atmospheric CH_4, atmMOB are likely to benefit from CH_4 produced by methanogenesis during anoxic conditions in deeper soil layers, and anoxic microsites in oxic soil [62]. Nevertheless, the utilization of alternative energy sources may have been an important driver for atmMOB evolution, considering the low atmospheric CH_4 concentrations in present, preindustrial and earlier times [63,64]. All four known atmMOB in pure culture, *M. rosea* SV97, *M. gorgona* MG08, *M. palsarum* NE2 and *M. aurea* KYG, oxidize either atmospheric H_2 and/or CO in addition to CH_4 [19,20,65]. Correspondingly, the utilization of atmospheric H_2 and CO has been shown to act as energy sources for a large diversity of microorganisms in soils and other environments globally [66]. While CH_4 and H_2 oxidation by atmMOB corresponded with the presence of pMMO and hydrogenase (HYD) genes, the genes encoding carbon monoxide dehydrogenase (CODH) were only present in *M. gorgona* MG08, but not in the genome of the CH_4 and CO oxidizing *M. palsarum* NE2 and only partially in *M. rosea* SV97 [20]. This suggests that other enzymes can be responsible for CO oxidation, one possibility being CO oxidation by pMMO [67]. CO oxidation by pMMO may inhibit CH_4 oxidation by competitive binding to pMMO, similar to ammonia [35].

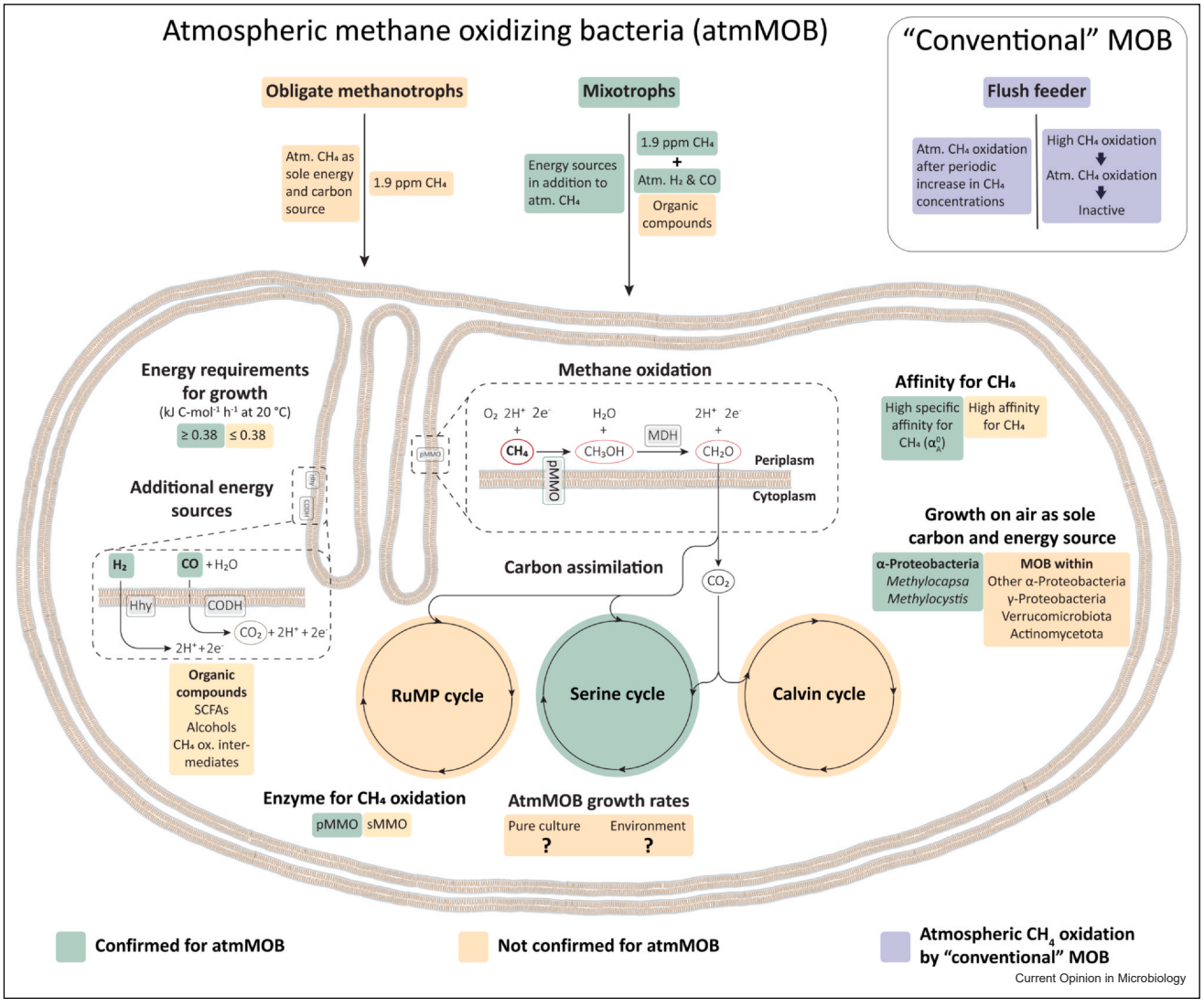
The observations of atmMOB utilization of H_2 and CO as alternative energy sources confirmed the hypothesis that mixotrophy could support atmospheric CH_4 oxidation, but the candidate molecules originally hypothesized as potential energy sources were short-chain fatty acids and alcohols [35]. Pratscher and colleagues investigated this by incubating forest soils with ^{13}C-acetate and trace concentrations of CH_4; they detected putative methanotrophic cells belonging to the USCα that were labeled with ^{13}C [68]. The finding of Pratscher and colleagues is an indication that atmMOB may harvest energy and carbon from sources other than trace gases. This is in line with the utilization of alternative energy sources such as acetate and ethanol by members of the genera *Methylocystis* and *Methylocapsa* [3], and methanol promoting atmospheric CH_4 oxidation by pure cultures and soils [69]. However, whether atmMOB can utilize acetate and alcohols at *in situ* concentrations to support growth remains unknown.

Closely related to the question of energy sources is how atmMOB harvest carbon for growth. Different carbon assimilation pathways have different efficiencies, and thus, the type of pathway may be important to explain the energy-limited growth on atmospheric CH_4. For example, the Calvin cycle is a less efficient route of carbon assimilation than the serine cycle, which in turn is less efficient than the ribulose monophosphate (RuMP) pathway [70]. However, in *M. gorgona* MG08, *M. palsarum* NE2 and *M. rosea* SV97, it was the expression of serine cycle enzymes that was observed during growth on air [20]. This is in line with the serine cycle being a known characteristic of alphaproteobacterial methanotrophs [71]. Gammaproteobacterial atmMOB (USCγ) MAGs (metagenome-assembled genomes) were shown to encode several genes of both the serine cycle and the RuMP pathway [72], but as of yet, it is unresolved how USCγ assimilates carbon.

Physiological diversity of atmMOB

Many clades were indicated by molecular environmental surveys to be atmMOB, and although most of these clades have not yet been confirmed to persistently oxidize atmospheric CH_4, the surveys do indicate considerable diversity of atmMOB. What this means in terms of functional diversity is still unknown, but even among the four closely related confirmed atmMOB, differences in substrate preferences, whole cell CH_4 oxidation kinetics, energy yields and protein expression patterns were found [20]. We propose that a variety of physiologies contribute to the biological sink for atmospheric CH_4, ranging from mixotrophic atmMOB that can grow with and without CH_4 and obligate atmMOB that are entirely dependent on CH_4 for growth, to flush-

Figure 3



A summary of knowledge and knowledge gaps related to methanotrophic lifestyles that contribute to atmospheric methane oxidation. Methanotrophs that can sustain uptake of atmospheric methane indefinitely, and wholly or partially support their energy and carbon needs for growth from atmospheric methane are referred to as atmospheric methane oxidizing bacteria (atmMOB). Green indicates experimentally confirmed properties of atmMOB. Yellow indicates unknown or hypothesized properties of atmMOB. Purple indicates how 'conventional' MOB have been shown to contribute to the atmospheric CH_4 sink. Abbreviations: ppm, parts per million; pMMO, particulate methane monooxygenase; sMMO, soluble methane monooxygenase; MDH, methanol dehydrogenase; atmMOB, atmospheric methane-oxidizing bacteria; CO, carbon monoxide; H_2, molecular hydrogen; CH_4, methane; CH_3OH, methanol; CH_2O, formaldehyde; kJ, kilojoule; C-mol, moles of carbon; SFCA, short-chain fatty acid.

feeding methanotrophs that can only sustain atmospheric CH_4 oxidation for a limited time after stimulation by high CH_4 concentrations.

Summary and conclusion

In Figure 3, we have summarized the current knowledge about atmMOB physiology and some of the most important knowledge gaps. To identify the capacity of atmMOB for CH_4 filtration and biomass production at low CH_4 concentrations, how their growth can be stimulated, and how their environmental dynamics will develop in response to climate change, we think that the following three actions are particularly important: 1. Identify the growth rates of atmMOB in pure culture and nature, and their response to changing energy input; 2. Identify whether compounds such as short-chain fatty acids, alcohols and sugars supply energy or carbon for their growth; 3. Identify how changes in the chemical (e.g. nitrogen availability) and physical (e.g. temperature) environment of atmMOB influence their growth.

Data Availability

No data were used for the research described in the article.

Declaration of Competing Interest

I hereby confirm that we declare no conflict of interest.

Acknowledgements

This study was supported by the Research Council of Norway FRIPRO Young Researcher Grant 315129, Living on Air, to ATT.

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- •• of outstanding interest

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