Applied Microbiology and Biotechnology (2024) 108:60
https://doi.org/10.1007/s00253-023-12978-3

MINI-REVIEW

Recent findings in methanotrophs: genetics, molecular ecology, and biopotential

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Received: 30 August 2023 / Revised: 8 September 2023 / Accepted: 1 October 2023

Abstract

The potential consequences for mankind could be disastrous due to global warming, which arises from an increase in the average temperature on Earth. The elevation in temperature primarily stems from the escalation in the concentration of greenhouse gases (GHG) such as CO_2, CH_4, and N_2O within the atmosphere. Among these gases, methane (CH_4) is particularly significant in driving alterations to the worldwide climate. Methanotrophic bacteria possess the distinctive ability to employ methane as both as source of carbon and energy. These bacteria show great potential as exceptional biocatalysts in advancing C1 bioconversion technology. The present review describes recent findings in methanotrophs including aerobic and anaerobic methanotroph bacteria, phenotypic characteristics, biotechnological potential, their physiology, ecology, and native multi-carbon utilizing pathways, and their molecular biology. The existing understanding of methanogenesis and methanotrophy in soil, as well as anaerobic methane oxidation and methanotrophy in temperate and extreme environments, is also covered in this discussion. New types of methanogens and communities of methanotrophic bacteria have been identified from various ecosystems and thoroughly examined for a range of biotechnological uses. Grasping the processes of methanogenesis and methanotrophy holds significant importance in the development of innovative agricultural techniques and industrial procedures that contribute to a more favorable equilibrium of GHG. This current review centers on the diversity of emerging methanogen and methanotroph species and their effects on the environment. By amalgamating advanced genetic analysis with ecological insights, this study pioneers a holistic approach to unraveling the biopotential of methanotrophs, offering unprecedented avenues for biotechnological applications.

Key points

- *The physiology of methanotrophic bacteria is fundamentally determined.*
- *Native multi-carbon utilizing pathways in methanotrophic bacteria are summarized.*
- *The genes responsible for encoding methane monooxygenase are discussed.*

Keywords CH_4 fluxes · Greenhouse gas · Methane oxidation · Methanotrophic bacteria

Introduction

Global warming and initiation of ice ages are still a serious scientific problem (Supran et al. 2023). It seems that the global warming debate is increasing following the publication of Cheng et al. (2022) which was emphasized on imbalance evidence of human influence on climate. This leads to considerable controversy among different environmental and atmospheric scientists, as evidenced in several previous publications (Lackner 2015; Harvey et al. 2023; Jansson and Wu 2023). These publications and reports questioned the relationship between the increasing concentration of greenhouse gases (GHG) and the earth surface temperature.

It is reported that the atmospheric CO_2 concentration was reached to 420 µmol mol^{-1} in 2022 (Le and Lee 2022) which was increased by 51% and is responsible for 16% of the greenhouse effect (Jang et al. 2023). It is demonstrated that the atmospheric CO_2 concentration is an important factor in global warming and climate change

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Published online: 06 January 2024

(Giunta et al. 2022). However, methane (CH_4) is a serious GHG that is 28 to 34 folds stronger than CO_2 in increasing global warming potential and climate change during the time (Wang et al. 2022). Le and Lee (2022) reported that the global atmospheric CH_4 concentration is increasing by 166% higher than the preindustrial level. Sadeh et al. (2023) reported that the CH_4 atmospheric concentration increased at a rate of 0.003 µmol mol^{-1} $year^{-1}$.

GHG cycling is complex and several biological and anthropogenic processes are involved (Lackner et al. 2021; Han et al. 2023). The most important anthropogenic activities are agriculture, fossil fuels, and biofuel burning, energy, waste, and industrial production (Chew et al. 2023). Previous research (Bartosiewicz et al. 2023) reported that waste, energy, industry, and agriculture contribute 20.61%, 28.65%, 0.10%, and 50.63% of the calculated annual CH_4 emission, respectively. The main biological source of atmospheric CH_4 in anoxic environments is produced by methanogens bacteria (Sadeh et al. 2023). The effect of reduction of CH_4 in reducing the global warming is 20 to 60 times more than CO_2 emissions (Sadeh et al. 2023). So, finding a solution way to reduce the atmospheric CH_4 is so critical.

Methanotrophs or methane-oxidizing bacteria (MOB) have the unique ability to use CH_4 as their sole carbon and energy source (Cheng et al. 2022). In this comprehensive review, we will focus on methanotroph bacteria, their taxonomy, physiology, diversity, ecology and distribution, associations of methanotrophs with other bacteria, biodegradation of toxic chemicals by methanotrophic bacteria, anaerobic methane oxidation mechanism, and factors affecting their function all based on the recent new findings and research all over the world.

In this comprehensive review, we addressed most recent findings in methanotrophs, including aerobic and anaerobic methanotrophs, taxonomy, diversity, genetics, molecular ecology, and their biochemical potential all based on the recent new findings and research all over the world. Although there are some previous reports on methanotrophs, however, this ground-breaking comprehensive review unveils a multitude of recent discoveries in the realm of methanotrophs, ranging from their intricate genetics to the dynamic landscape of molecular ecology. With a focus on recent genetic revelations, molecular intricacies, and untapped biopotential, this research introduces a novel chapter in methanotrophic studies, underscoring its contribution to both fundamental knowledge and practical applications. By amalgamating advanced genetic analysis with ecological insights, this study pioneers a holistic approach to unraveling the biopotential of methanotrophs, offering unprecedented avenues for biotechnological applications.

Methanotrophic bacteria, aerobic, and anaerobic

Methanotrophic bacteria are a subgroup of so-called methylotrophic bacteria (methylotrophs), and they can utilize methane as their sole carbon and energy source (while the latter metabolize methanol). Methanotrophs can catalyze the oxidation of methane to methanol by using methane monooxygenases (MMOs) enzyme (Rhee et al. 2019; Lackner et al. 2022). It is well known that the aerobic methanotrophs are Gram-negative bacteria and with a total of 23 genera and roughly 60 species being identified (Guerrero-Cruz et al. 2021; Wang et al. 2023a). There are different types of aerobic methanotrophs known as Gammaproteobacteria, type I, with families *Methylococcaceae* and *Methylothermaceae*, and Alphaproteobacteria, type II, with families *Methylocystaceae* and *Beijerinckiaceae* (Guerrero-Cruz et al. 2021).

The metabolism and unique pathways of methanotroph bacteria including an important role of formaldehyde as an intermediate in catabolism and anabolism are shown in Fig. 1 (Hanson and Hanson 1996). There is another pathway (dihydroxyacetone) in yeast strains which is growth on methanol and leads to formaldehyde assimilation. Then, carbon assimilation will be occurred after oxidation steps (Giunta et al. 2022).

The anaerobic methanotrophs were found in the marine sediments for the first time where sulfate is present and is involved in methane consumption (Kalyuzhnaya et al. 2019). Sulfate-reducing bacteria (SRB) are responsible for sulfate-dependent anaerobic methane oxidation process in anaerobic conditions (Yu et al. 2022) (Fig. 2). However, it is reported that nitrite (NO_2^-) and nitrate (NO_3^-) are more abundant than sulfate (SO_4^{2-}) in freshwater environments and can

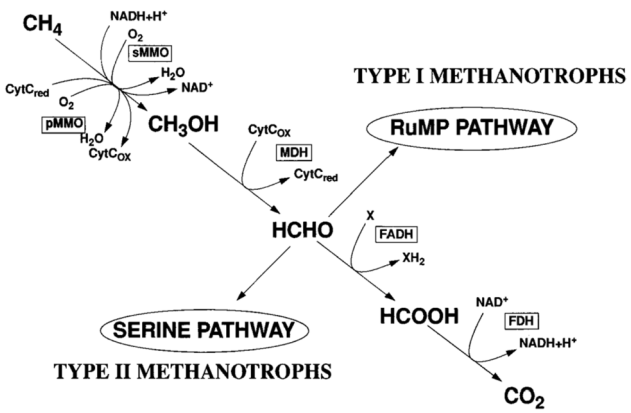

Fig. 1 Routes for the conversion of methane through oxidation and the incorporation of formaldehyde. Short forms: CytC, cytochrome c; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase (Hanson and Hanson 1996)

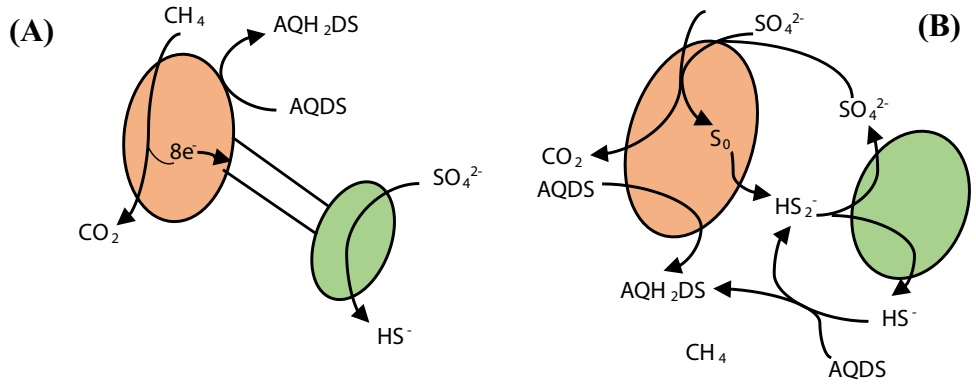

Fig. 2 Diagram illustrating two theoretical situations of anaerobic methane oxidation linked to AQDS as the final electron receptor by ANME-2 and SRB communities. The ANME-2 cell is depicted in red, while the cooperating SRB partner is shown in green. In scenario **A**, ANME oxidizes methane and conducts extracellular electron transfer to reduce AQDS. While sulfate boosts ANME metabolism, it is not respired, and the synergistic SRB remain inactive. In scenario **B**, ANME oxidizes methane and reduces both AQDS and sulfate. Subsequently, SRB mediate the transformation of zerovalent sulfur, a by-product of ANME, into various compounds. This scenario suggests a concealed sulfur cycle where AQDS and sulfide chemically react to regenerate zero-valent sulfur. ANME's metabolic activity intensifies due to the availability of these two electron acceptors. Our research findings align with the extracellular electron transfer concept depicted in scenario **A**, rather than the active sulfate respiration and sulfur disproportionation model shown in scenario **B** (Yu et al. 2022)

involve in microbial processes of methane oxidation (Malyan et al. 2021). Equation 1 summarizes the sulfate reduction involved in anaerobic methane oxidation processes (Siniscalchi et al. 2022):

$$CH_{4\ (aq)} + SO_4^{2-} \rightarrow HS^- + HCO_3^- + H_2O \qquad \Delta G = -34\ kJ\ mol^{-1}\ CH_{4\ (l)} \tag{1}$$

The co-occurrence of anaerobic methanotrophic archaea and *Deltaproteobacteria* was reported in sulfate-reducing enrichment environments, and it is probably in a syntrophic manner (Siniscalchi et al. 2022); they both have an extremely low energy yield of the net reaction (Eq. 1). However, the biological nature of sulfate reduction bacteria is complex and is a geomicrobiological puzzle.

Several documents demonstrated the effective role of nitrate/nitrite reduction in methane oxidation that is critical to processes of GHG emission (Baba and Miyaji 2020; Farhan Ul Haque et al. 2020; Hwang and Lee 2023). Recent findings in molecular biology techniques could successfully determine the important role of nitrate/nitrite reduction bacteria and archaea in methane oxidation (N-AOM processes) (Le and Lee 2023). In this context, novel biological 16S rRNA and gene amplicon analysis demonstrated that *Methylomirabilis*- and *Methanoperedens*-like microorganisms are more widespread than previously believed (Hopple et al. 2022). Meanwhile, there are new evidence that novel *Candidatus Methylomirabilis sinica* and *Candidatus Methylomirabilis lanthanidiphila* can support the N-AOM process (Hwang and Lee 2023; Bhattarai et al. 2019). Presence of *M. nitroreducens* and *M. oxyfera* require the controlled environmental conditions because of their slow growth rate of these bacteria (Wang et al. 2022).

Isotopic evidence demonstrated the important role of *M. nitroreducens* as a N-AOM using nitrate as electron acceptor (Le et al. 2021). As shown in Fig. 3, methanogenesis is a biological process that consumes CH_4 and produces CO_2 and H_2 using methyl coenzyme M reductase (MCR). It is recognized that some new genes are involved in MCR synthesis such as mcrABCDG genes in *M. nitroreducens.* So, mcrA can be used as an important biomarker to identify methanotrophic bacteria in different cultures (Chan and Lee 2019). The nitrate reductase genes and reverse methanogenesis were recognized in the genome of *M. nitroreducens*, whereas no enzymes were reported for the subsequent denitrification. So, some anaerobic methanotrophic archaea can reduce NO_3^- to NO_2^- and must rely on a partner to further reduce NO_2^- to N_2 (Muñoz-Gómez et al. 2022).

The most common operating conditions set in N-AOM investigations are summarized in Table 1. This information is so important on future research for the N-AOM under relevant conditions. It should be mentioned that the CH_4 consumption was not reported in different N-AOM studies which complicates the evaluation of the reported process performance. In this context, N_2 production data cannot strongly demonstrate the N-AOM occurrence, because alternative electron donors are present in the inoculum which are able to trigger nitrite/nitrate reduction.

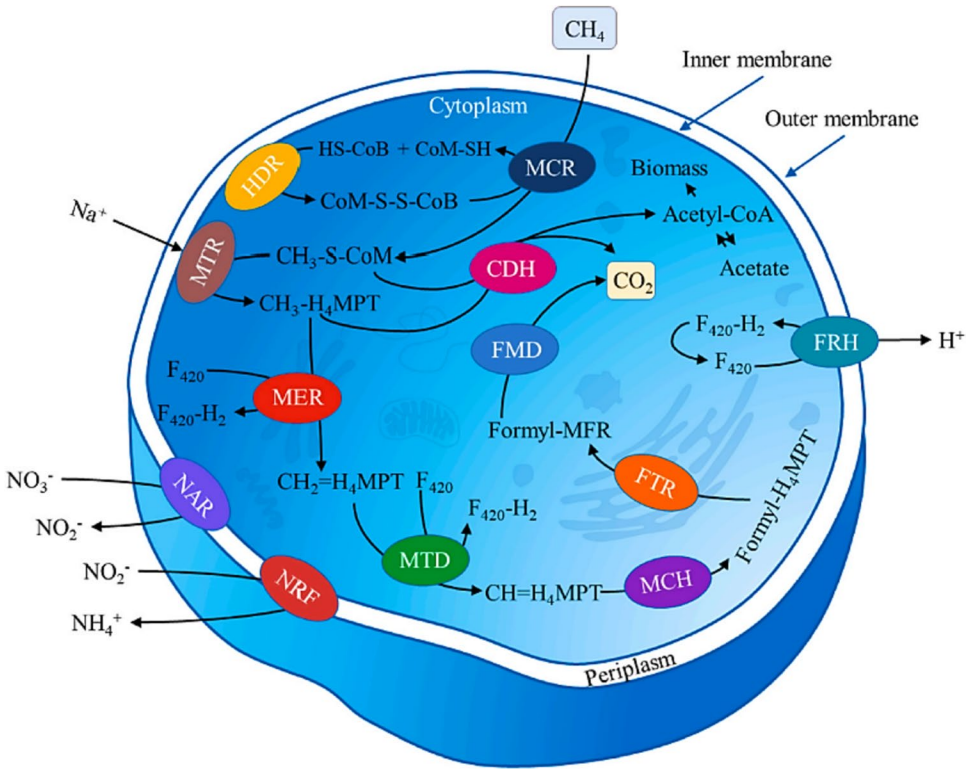

Fig. 3 The reverse methanogenesis pathway in *M. nitroreducens* linked with nitrate reduction involves a series of enzymes: CDH (molybdenum-dependent formylmethanofuran dehydrogenase), FMD (formylmethanofuran dehydrogenase), FRH (F420-dependent hydrogenase), FTR (formylmethanofuran-H4MPT formyltransferase), HDR (coenzyme B-coenzyme M heterosulfide reductase), MCH (methenyl-H4MPT cyclohydrolase), MCR (methyl coenzyme M reductase), MER (methylene H4MPT reductase), MTD (methylene-H4MPT dehydrogenase), MTR (methyl-H4MPT:coenzyme M methyltransferase), NAR (nitrate reductase), and NRF (nitrite reductase ammonium-forming). Within this context, CoA, CoB, and CoM represent coenzymes A, B, and M, respectively. H4MPT corresponds to tetrahydromethanopterin, and MFR signifies methanofuran (Costa et al. 2022)

Taxonomy of methanotrophic bacteria

Challenges encountered in the pursuit of obtaining pure cultures, understanding phenotypic traits, and adhering to the guidelines outlined in the *International Code of Nomenclature of Bacteria* (Oren 2020) have given rise to limitations in the classification of methanotrophs. Over the past five decades, a considerable number of methanotrophic cultures have been successfully isolated and formally characterized, beginning with the seminal work of Whittenbury. Presently, our understanding encompasses 18 clusters of aerobic methanotrophic bacteria within the *Gammaproteobacteria* class and an additional five clusters within the *Alphaproteobacteria* class, constituting a diverse spectrum of approximately 60 distinct species.

Methanotrophic organisms with formally recognized names are classified into two distinct classes, namely, the *Gammaproteobacteria* (also referred to as type I and type X) and *Alphaproteobacteria* (also referred to as type II) classes (Muñoz-Gómez et al. 2022). Gammaproteobacterial methanotrophs are categorized within the *Methylococcales* order and encompass three families: *Methylococcaceae*, *Methylothermaceae*, and *Crenotrichaceae*. This group presently encompasses 42 species with officially published names distributed across 19 genera (Le et al. 2021). The *Methylothermaceae* family encompasses two genera and two species, while the *Crenotrichaceae* family comprises a single genus and species (Nguyen and Lee 2021). These taxonomic assignments are primarily founded on the phylogenetic analysis of *16S rRNA* gene sequences (Cheng et al. 2022). A summary of discernible characteristics displayed by species within *Methylomicrobium*, *Methylotuvimicrobium* gen. nov., and *Methylosarcina* can be found in Table 2.

Category I methanotrophs have been classified into three distinct groups. The initial group, comprising phenotypes 1 to 3, is represented by the species *Methylomonas methanica*, *Methylomonas fodinamm*, and *Methylomonas aurantiaca*, which are characterized by the presence of pink and orange carotenoid pigments. These species share common attributes, including encapsulated cells with a coccobacillary or rod-shaped morphology, the presence of poly-P-hydroxybutyrate inclusions, non-desiccation resistant cysts, carotenoid

Table 1 Performance of N-AOM processes at different experimental conditions

Inoculum	Liquid-phase operation	Gas-phase operation	NO_2^- removal rate ($g\ m^{-3}\ h^{-1}$)	NO_3^- removal rate ($g\ m^{-3}\ h^{-1}$)	CH_4 removal rate ($g\ m^{-3}\ h^{-1}$)	Reference
Sludge from a WWTP	Batch	Batch	NE	1.32×10^{-3}	NE	Islas-Lima et al. 2004
Anoxic sediment from a canal	Batch	Batch	1.4	NE	0.21	Costa et al. 2022
Sediments from ditches draining agricultural land of the river Rhine	Semicontinuous	Continuous	1.22	NE	0.70	Ettwig et al. 2009
Mixture of sediments from a freshwater lake, anaerobic digester sludge, and the return sludge	Semicontinuous	NE*	NE	7.0	NE	Hu et al. 2009
Secondary sludge from an activated sludge system	Semicontinuous	Continuous	0.40	NE	0.19	Luesken et al. 2011
Sludge from a parental reactor	Batch and semi-continuous	NE	0.1	NE	NE	Hu et al. 2011
Sludge from a parental reactor	Semicontinuous	Continuous	NE	7.9	NE	Shi et al. 2013
Mixture of methanogenic sludge and activated sludge	Batch	Batch	NE	2.82	NE	Ding et al. 2014
Mixture of secondary sludge and digested secondary sludge	Continuous	Continuous	1.5	NE	NE	Kampman et al. 2014
Coastal sediments	Semicontinuous	Batch	9.15	NE	0.27	He et al. 2015
Sediments from a freshwater lake	Batch	Batch	2.1	2.5	0.42	Fu et al. 2017
Mixture of fresh secondary activated sludge and anoxic sludge from a denitrifying bioreactor	Continuous	Continuous	NE	2.8	0.03–20.6	Lopez et al. 2017
Sludge from a parental reactor	Continuous	Continuous	NE	7.1	NE	Liu et al. 2019
Secondary activated sludge	Semicontinuous	Continuous	NE	1.4–9.6	21–55	Valenzuela et al. 2021

*NE, not evaluated

pigments, and motility facilitated by a single polar flagellum. Additional distinguishing phenotypic traits for these species are outlined in detail in Table 3.

Type II methanotrophs pertain to the *Alphaproteobacteria* class and encompass representatives from the *Methylocystaceae* and *Beijerinckiaceae* families. Within the type II methanotrophs, there are closely affiliated clusters present in the validated species within the *Methylocystis* and *Methylosinus* genera. Specific characteristics that differentiate the genera of type II methanotrophs are outlined in Table 4. On the other hand, type I methanotrophs are categorized as the "high capacity" group, primarily inhabiting environments abundant in methane but lacking in oxygen. Conversely, the "low capacity" type II methanotrophs prevail in environments with scant methane and ample oxygen. This distinction implies that varying types of methanotrophs

Table 2 Phenotypic characteristics of member species of *Methylomicrobium*, *Methylotuvimicrobium* gen. nov. (Orata et al. 2018)

Genus	*Methylomicrobium*			*Methylotuvimicrobium gen. nov.*				
Species	*Methylomicrobium agile*	*Methylomicrobium album*	*Methylomicrobium lacus c.n.*	*Methylotuvimicrobium alcaliphilum c.n.*	*Methylotuvimicrobium buryatense c.n.*	*Methylotuvimicrobium japanense c.n.*	*Methylotuvimicrobium kenyense c.n.*	*Methylotuvimicrobium pelagicum c.n.*
Former name	*NA*	*NA*	*Methylosarcina lacus*	*Methylomicrobium alcaliphilum*	*"Methylomicrobium buryatense"*	*Methylomicrobium japanense*	*Methylomicrobium kenyense*	*Methylomicrobium pelagicum*
Type strain	ATCC 35068	BG8	LW14	20Z	5B	NI	AMO1	AA-23
Type species	Yes	No	No	Yes	No	No	No	No
Characteristics								
Pigmentation	W to SL	W to SL	W to SL	W to SL	W to SL	W to SL	W to SL	W to SL
Motility	+	+	-	+	+	+	+	+
Cyst formation	-	-	-	-	-	-	-	-
Desiccation resistance	-	-	-	+	+	-	-	-
Growth occurs with 3.0% NaCl	-	-	NR	+	+	+	+	+
Temp. growth, range (°C)	10–37	10–37	4–35	NR	4–45	NR	NR	10–30
Temp. growth, optimum (°C)	25–30	25–30	28–30	NR	28–30	15–37	NR	20–25
Growth at 37 °C	+	V	-	+	+	+	-	-
Growth at 45 °C	-	-	NR	-	+	-	-	-
Heat resistance (80 °C)	-	-	-	+	+	-	-	-
pH growth, range	6–9	6–9	4–7	6.5–9.5	6–11	NR	9–10.5	6–8.5
pH growth, optimum	7	7	5.5–6.5	9	8.5–9.5	8.1	10	7
pMMO	+	+	+	+	+	+	+	+
sMMO	-	-	-	-	+	+	-	-
Main fatty acid	C16:1 ω5t	C16:1 ω5t	C16:1 ω8c	C16:1 ω7c	C16:1 ω7c	C16:1	NR	C16:1 ω5t
DNA G + C content (mol%)	58.1–59.6	54.4–56.3	52–54.7	48–49	48–49	49	50.2	48.5

may partake in methane oxidation under diverse moisture conditions, as highlighted by Zhou et al. (2020).

Biotechnological potential of methanotrophs

Methanotrophs, found widespread, offer potential for remediating contaminated sites. A research has demonstrated that introducing methane can enhance the aerobic breakdown of halogenated hydrocarbons, as discussed in the review by Le et al. (2021). Both variants of the MMO can convert these halogenated compounds. However, the available data suggests that despite its slower degradation rate, the particulate MMO (pMMO) ultimately proves to be the more efficient system. Considering this, the utilization of adaptable *Methylocystis* strains becomes appealing. Within these strains, the pMMO is activated in the presence of acetate or ethanol, as observed in studies by Khanongnuch et al. (2022). This approach could be employed to provide the necessary reducing agents for the MMO. Such a strategy would be more feasible to implement in polluted sites compared to methane introduction and would additionally prevent competition for binding to the monooxygenase enzyme.

As another example of bioremediation, *Methylocella* was among the bacteria associated with degradation of plastics in landfill lysimeters (Khanongnuch et al. 2022). Obligate methanotrophs such as *Methylococcus capsulatus*, which can

Table 3 Phenotypic characteristics of methanotrophic phena defined by numerical analysis (sources: Zhou et al. 2020; Khanongnuch et al. 2022; Priyadarsini et al. 2023)

Characteristic	Phenon									
	1: Methylomonas methanica	*2: Methylomonas fodinarum*	*3: Methylomonas aurantiaca*	*4: Methylococcus* spp.	*5: Methylomonas* spp.	*6: Methylomonas agile*	*7: Methylomonas alba*	*7: Methylomonas luteus*	*8: Methylococcus luteus*	*9: Methylomonas pelagica*
Cell morphology										
Cocci	-	-	-	+	+	+	+	+	+	+
Ellipsoidal	-	-	-	+	+	+	87	50	50	+
Rods	+	+	+	-	-	50	25	-	-	-
Coccibacilli	+	+	+	33	+	+	+	-	-	+
Reniform or vibrioid	-	-	-	-	-	-	-	-	-	-
Pyriform	-	-	-	-	-	-	-	-	-	-
Length 0.5–1.0 pm	77	+	+	-	67	50	37	-	-	+
Length 2.1–3.0 pm	92	+	+	+	+	+	+	+	+	+
Length 2.1–3.0 pm	-	-	-	33	-	-	-	-	-	-
Width c0.5 pm	8	-	-	-	-	50	-	-	-	+
Width 0.6–1.0 pm	92	+	+	+	+	+	+	+	+	+
Poly-P-hydroxybutyrate	+	+	+	+	+	50	+	+	+	+
Polyphosphate	+	+	11	17	-	+	87	-	-	-
Cyst formation	+	+	+	+	67	-	-	+	+	-
Exospore formation	-	-	-	-	-	-	-	-	-	-
Type II intracytoplasmic membrane	+	+	+	+	+	+	+	+	+	+
Type II intracytoplasmic membrane	-	-	-	-	-	-	-	-	-	-
Capsule	69	+	+	+	+	+	50	25	25	-
Motility (1 polar flagellum)	+	+	+	+	+	+	+	-	-	+
Motility (> 1 polar flagella)	-	+	-	-	-	-	-	-	-	-
Spinae	-	-	-	-	-	-	-	-	-	-
Chain formation	23	-	+	-	-	-	-	25	25	-
Colony morphology										
Translucent	-	-	-	-	-	+	13	-	-	+
Opaque	+	+	+	+	+	-	87	+	+	+
Low convex	+	+	93	67	+	+	+	+	+	-
High convex	38	-	82	-	-	-	-	-	-	+
Entire edges	+	+	+	+	+	50	+	+	+	-
Lobate or irregular edges	-	-	-	-	-	50	66	50	50	+
Butyrous consistency	62	-	-	+	+	+	+	+	+	-
Viscid or mucoid consistency	62	+	79	17	-	-	-	25	25	38
Cartilaginous consistency	38	-	61	-	-	-	-	-	-	+
White or buff	-	-	-	+	67	+	+	-	+	+

Table 3 (continued)

Characteristic	Phenon									
	1: Methylomonas methanica	*2: Methylomonas fodinarum*	*3: Methylomonas aurantiaca*	*4: Methylococcus* spp.	*5: Methylomonas* spp.	*6: Methylomonas agile*	*7: Methylomonas alba*	*7: Methylomonas luteus*	*8: Methylococcus luteus*	*9: Methylomonas pelagica*
Brown	-	-	-	-	63	-	-	-	-	-
Pink or red	+	-	-	-	-	-	-	-	-	-
Orange	-	+	+	-	-	-	-	-	-	-
Yellow	-	-	-	-	-	-	-	+	-	-
Brown (diffusible)	-	-	-	+	+	-	-	-	-	-
Yellow (diffusible)	-	-	-	-	-	-	-	25	-	-
Carotenoids	+	+	+	-	-	-	-	-	-	-
Growth in static liquid culture										
Evenly dispersed	-	-	-	+	+	+	+	+	+	+
Surface pellicle	+	+	+	-	-	-	-	-	-	-
Growth at:										
pH 9.0	92	91	+	+	+	50	+	+	-	-
pH 5.5	23	82	+	+	+	-	38	+	+	+
pH 5.0	-	-	89	33	-	-	-	25	-	-
20 °C	+	+	+	+	67	+	+	+	-	-
28 °C	+	+	+	+	+	+	+	+	-	25
37 °C	15	-	+	67	+	+	75	75	-	+
45 °C	-	-	-	-	-	-	-	-	-	+
55 °C	-	-	-	-	-	-	-	-	-	75

Table 4 Characteristics that distinguish genera of type II methanotrophs

Characteristics	*Methylosinus*	*Methylocystis*
Cell morphology	Vibriod or pyriform	Cocci, curved rods, ellipsoidal
Cyst formation (desiccation sensitive)	-	+
Exospore (bud) formation	+	-
Lysed by 2% (wt/vol) SDS	+	-
Representative species	*M. trichosporium* *M. sporium*	*M. echinoides, M. parvys, M. pyriformis, M. minimus*

All these organisms are characterized as gram-negative, strictly aerobic, and obligate methylotrophs. They utilize the serine pathway to assimilate formaldehyde. Notably, they possess intracytoplasmic membranes that are oriented parallel to the cell wall. Their growth is not supported at a temperature of 458 °C. These microorganisms possess a complete tricarboxylic acid cycle but lack the enzymes associated with the Calvin-Benson cycle. Additionally, they engage in nitrogen fixation through an aerotolerant nitrogenase system. The prominent phospholipid fatty acid is 18:1v8c, and their DNA base compositions exhibit a range of 62 to 67 mol% G1C (Whiddon et al. 2019; Le et al. 2021)

grow relatively quickly and to high cell densities, have been exploited for production of single-cell protein (Khanongnuch et al. 2022). While *Methylocella*, which exhibits slower grow, may not be as useful to produce low value, bulk chemicals, it can still be grown to high cell densities in fermenter culture (Priyadarsini et al. 2023). Its metabolic versatility warrants further examination in this respect. Large-scale production of methanol from methane is an attractive proposition and promising results have been obtained in several studies (Baba and Miyaji 2020). As an illustration, combinations of *Methylomonas methanica* and *Methylocella tundra* co-cultures, which were encapsulated within silica gel, were supplied with simulated biogas as a nutrient source.

It is worth noting that the introduction of hydrogen resulted in a significant enhancement in methanol production, nearly doubling it to approximately 0.32 g l^{-1}, with a corresponding 66% conversion efficiency (Zhou et al. 2020). However, a notable challenge in utilizing the MMO for methanol production lies in the need for a costly electron donor, such as formate, to facilitate methane oxidation. Nonetheless, a promising solution may be found in facultative methanotrophs, as they possess the capability to utilize compounds like acetate, which are commonly found in waste streams, as an alternative electron donor (Lee et al. 2023).

While *Methylocella* spp. exhibit a wider array of metabolic capabilities compared to obligatory methanotrophs, their potential biocatalytic applications have not been fully explored. The soluble methane monooxygenase (sMMO) has traditionally been recognized as an exceptionally versatile biocatalyst, facilitating the oxidation of a diverse spectrum of compounds, including alkanes, alkenes, and even relatively large aromatics like naphthalene (Priyadarsini et al. 2023).

The utilization of intact cells of methanotrophs like *Methylococcus capsulatus* for the production of chemicals such as propylene oxide (from propylene) is a viable approach. However, the toxicity associated with this metabolite necessitates the implementation of a recycling system to regenerate the entire-cell biocatalyst (Samanta and Sani 2023). If *Methylocella* demonstrates reduced susceptibility to the adverse effects of propylene epoxide, it could potentially have an advantage over *M. capsulatus* in the production of this compound. This advantage stems from *Methylocella* ability to harness alternative energy sources for facilitating the oxidation of propylene through the sMMO enzyme.

Beyond *Methylocella* metabolic adaptability, these strains hold a distinct advantage over obligate methanotrophs, as the expression of the sMMO is not inhibited by copper (Cruz and Pijuan 2022). The potential utilization of sMMO as a biocatalyst, in conjunction with the ability to use multicarbon compounds like succinate or acetate for both carbon and energy, suggests that *Methylocella* could emerge as a promising cellular platform for the production of valuable commodities. Notably, this includes the production of valuable substances such as chiral alcohols and epoxides.

Findings on physiology of methanotrophic bacteria

Gaining insights into the factors governing methane metabolism and the ecological behavior of methanotrophic bacteria necessitates a comprehensive grasp of the physiological traits exhibited by diverse methanotroph groups. Distinctions in the enzyme systems utilized by various genera and species to catalyze methane oxidation, the pathways employed for assimilating one-carbon units into central metabolic processes, the chemical constitution of cellular components, regulatory mechanisms governing one-carbon compound metabolism, and the nutritional responses of distinct methanotrophs collectively determine their competencies in varying habitats. Hence, it is apt to assess the present understanding of the physiological attributes characterizing different methanotroph groups, particularly in relation to their aptitude for thriving, proliferating, and methane oxidation

across diverse environments. Additionally, this assessment should consider their potential for breaking down hazardous environmental contaminants that pose risks to human health and ecosystem stability.

Methane oxidation

The initial step in the oxidation of methane by aerobic methanotrophs is catalyzed by MMOs. These MMOs (Bo et al. 2023; Samanta and Sani 2023) represent classical monooxygenases that employ two reducing equivalents to cleave the O-O bonds of dioxygen, a process also noted by Lee et al. (2023) and Zhou et al. (2023). Within this process, one of the oxygen atoms undergoes reduction, ultimately resulting in the production of water (H_2O), while the other oxygen atom is integrated into methane, leading to the formation of methanol (CH_3OH). Two distinct forms of MMOs have been identified in methanotrophic bacteria (Shen et al. 2023). It is well-established that in most *Proteobacteria* and some *Verrucomicrobia*, the process of methane oxidation is linked to the creation of specialized subcellular compartments (Khanongnuch et al. 2022).

One of these forms, known as sMMO, employs NADH + H^+ as an electron donor and retains its solubility even after centrifugation of cell extracts at 150,000 × g for 75 min, as detailed by Singh et al. (2023). It is widely acknowledged that all sMMOs consist of three distinct components. The first component, the hydroxylase, possesses a size of 245 kDa and contains nonheme iron. This hydroxylase is comprised of three different subunits, namely, a, b, and g. The second component, referred to as the B component, has a mass of 15.8 kDa, lacks any cofactors, and appears colorless. The third component, known as the reductase component, boasts a size of 38.4 kDa and includes both flavin adenine dinucleotide and an Fe_2S_2 cluster. For a more in-depth understanding of this enzyme, its reaction kinetics, and the individual roles of each component in the catalytic cycle, comprehensive reviews are available elsewhere (Han et al. 2023).

Oxidation of formaldehyde and formate

The majority of the necessary reducing potential required for methane metabolism is generated through a series of steps involving the oxidation of formaldehyde, progressing through formate, and ultimately leading to the production of carbon dioxide. Within methylotrophs, there are multiple enzyme systems responsible for the oxidation of formaldehyde to formate (Rozova et al. 2021). These systems encompass NAD (P)-linked aldehyde dehydrogenases, which may or may not necessitate reduced glutathione or other cofactors, as well as dye-linked dehydrogenases, which are quantified through the reduction of dyes like 2,6-dichlorophenol, (Singh et al. 2023).

The conversion of formate to carbon dioxide is facilitated by an NAD-dependent formate dehydrogenase in most, if not all, methanotrophs (Schmitz et al. 2022). Some methylotrophs that employ the RuMP pathway for formaldehyde assimilation utilize a cyclic pathway for the oxidation of formaldehyde to carbon dioxide (Cruz and Pijuan 2022). In this pathway, formaldehyde and ribulose-5-phosphate engage in a reaction, yielding hexulose-6-phosphate, which is subsequently isomerized to fructose-6-phosphate, eventually transforming into glucose-6-phosphate. This glucose-6-phosphate undergoes further oxidation to yield 6-phosphogluconate. As the cycle progresses, 6-phosphogluconate is oxidized to generate both carbon dioxide and ribulose-6-phosphate, effectively concluding the cyclic pathway responsible for formaldehyde oxidation. NAD1 or $NADP^+$ serve as electron acceptors in the two oxidation steps within this cycle. While most obligatory methanotrophs utilize the linear pathway for formaldehyde oxidation, many non-methane utilizing methylotrophs predominantly employ the cyclic pathway (Khanongnuch et al. 2022).

Methanol oxidation

Methanol, originating from both endogenous sources (resulting from methane oxidation via MMO) and exogenous sources (such as pectin and lignin degradation), undergoes oxidation to formaldehyde via a periplasmic methanol dehydrogenase (MDH) in gram-negative methylotrophs (Le and Lee 2023). MDH exists as an $\alpha_2\beta_2$ tetramer, composed of large (60 to 67 kDa) and small (8.5 kDa) subunits (Le and Lee 2022). This enzyme is classified as a quinoprotein, with each tetramer containing 2 moles of pyrroloquinoline quinone and 1 mole of calcium (Zhu et al. 2022). The transfer of electrons from MDH to cytochrome c_L, an atypical cytochrome serving as the specific electron acceptor for MDH, is the subsequent step (Tikhonova et al. 2023). Cytochrome c_L, in turn, is oxidized by a typical class I cytochrome c (cytochrome c_H), which is also specific for methanol oxidation (Kang-Yun et al. 2022). Importantly, all three components—MDH, cytochrome c_L, and cytochrome c_H—are soluble and reside within the periplasm of gram-negative methylotrophs (Tentori et al. 2022). In contrast, gram-positive methylotrophs employ an NAD-linked MDH for methanol oxidation, while methanol-oxidizing yeast species use a methanol oxidase system for the same purpose. Notably, these enzymes have not been detected in gram-negative methanotrophic bacteria (Priyadarsini et al. 2023).

The regulation of MDH (malate dehydrogenase) synthesis involves complex regulatory networks in facultative methylotrophs and is at least present in one methanotrophic organism. A comprehensive understanding of the control mechanisms governing MDH synthesis has been extensively explored in previous literature (Howe et al. 2023; Venetz et al. 2022).

Findings on ecology and distribution of methanotrophic bacteria: 16S RRNA and functional genes

The most common habitats for methanotroph bacteria is shown in Fig. 4. Nearly all samples taken from muds, swamps, rivers, rice paddies, oceans, ponds, soils from meadows, deciduous woods, streams, sewage sludge, and several other environments contained methanotrophic bacteria (Oudova-Rivera et al. 2023). Estimations of methane emissions from wastewater treatment are scarce and accurate determinations are limited (Moore et al. 2023) (in detail in "Underestimation of Sector-Wide Methane Emissions from United States Wastewater Treatment"). When employing viable-count methods, the quantities of methanotrophs identified in soils, sediments, and aquatic environments spanned from 103 to 106 cells per gram (Zhou et al. 2020). Peltokangas et al. (2023) reported that significant amounts of CH_4 were produced even after homogenization of soil samples, where the anoxic microsites were destroyed. This has led to some authors to hypothesize that methanogens are not the sole source of CH_4 in oxic soils, but the possibility of non-microbial CH_4 formation in soils must be considered (Galera et al. 2023). Lin et al. (2023) conducted an experiment in which CH_4 release from soil increased with increasing temperature and organic C content, and with the addition of water to dried soils. Certain temperatures examined, reaching as high as 70 °C, exceeded the established enzymatic activity range of methanogens, effectively ruling out the likelihood of microbial methane (CH_4) production. This implies the presence of an unidentified chemical mechanism generating CH_4 in oxygen-rich soil environments. Notably, there has been recent documentation of the abiotic creation of CH_4 under extremely oxidizing conditions, and this phenomenon could have significance within soil ecosystems (Hopple et al. 2022).

Iqbal et al. (2023) highlighted the significance of rice paddies as a substantial source of atmospheric methane (CH_4), contributing to approximately 10% of total anthropogenic CH_4 emissions. In freshwater lakes, it is commonly assumed that high methane oxidation activity occurs at the

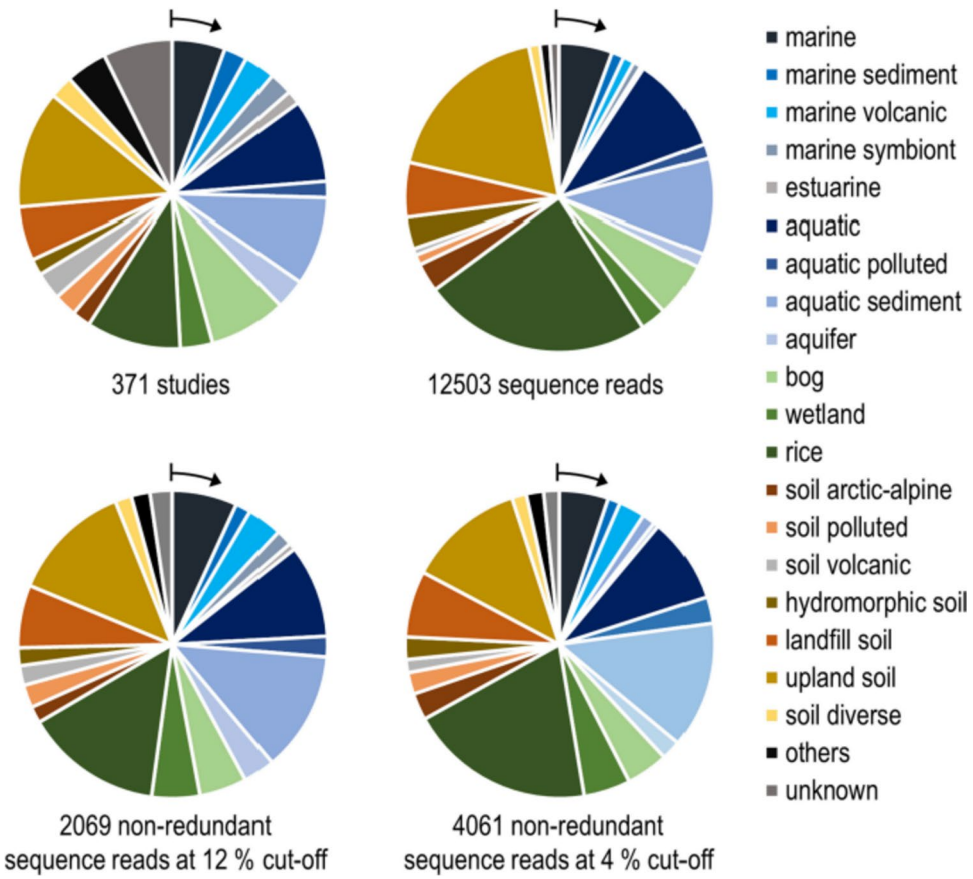

Fig. 4 The research studies investigated a certain number of habitats, as shown in the upper left diagram. The *pmoA* sequences from the NCBI database were categorized based on the environments in which they were identified, depicted in the upper right diagram. The lower diagrams, however, encompass non-redundant sequence reads only, excluding duplicate sequences found within the same study and in the same operational taxonomic unit (I). The directional arrows indicate the location of the group represented as the initial entry in the legend (Knief 2015)

oxic-anoxic transition zones (Bashir et al. 2023). An analysis of sediment microbial communities, based on *16S rRNA* gene sequencing, revealed the presence of diverse groups of aerobic methanotrophs, including type I gamma- and type II alpha-proteobacterial MOB (Wang et al. 2023b). Within the *Methylococcaceae* family, varying proportions were observed, ranging from 0.07 to 0.23% of the total, with the highest occurrence at a depth of 3 cm. In contrast, type II *alpha-MOB Methylocystis* was found at much lower relative abundances, peaking at 0.01% at a depth of 6 cm (Kaise et al. 2023).

It is noted that approximately 50–65% of total methane emissions are attributed to anthropogenic activities, encompassing ruminant husbandry, fossil fuel extraction and usage, rice paddy agriculture, and emissions from landfills and waste. This has led to a current atmospheric methane concentration increase of 2.5 times compared to preindustrial levels (IPCC 2013). Remarkably, scientists have enhanced their understanding of phylogenetic relationships and the discovery of new methanogens within rice fields by employing both conserved and functional gene sequences, including the *16S rRNA* and *mcrA* genes. Additional information on this subject is available in Table 5.

Findings on the native multi-carbon utilizing pathways in methanotrophic bacteria

In methanotrophic bacteria, both sMMO and pMMO can catalyze the hydroxylation of different alkanes. pMMO can only oxidize C1–C5 alkanes, in contrast to sMMO, which has a much wider substrate range (Chan and Lee 2019). These findings suggest that the substrate binding site only accepts straight-chain C1–C5 hydrocarbons in pMMO

Table 5 Description of novel methylotrophs and methanogens from different ecosystems

Novel microbes	Methanotrophs/Methanogens	Ecosystem	Isolation strategy	Reference
Candidatus Methylomirabilis	Methanotrophs	Paddy soil	Metagenomic approach	He et al. (2015)
Haliea sp. ETY-M	Methanotrophs	Marine	Conserved/functional gene-based phylogeny	Suzuki et al. (2012)
Halie asp. ETY-NAG	Methanotrophs	Marine	Conserved/functional gene	Suzuki et al. (2012)
Methanobacterium kanagiense	Methanogens	Paddy soil	Pureculture technique & polyphasic taxonomy	Kitamura et al. (2011)
Methanobacterium lacus AL-21	Methanogens	Acidic soil	Phylogeny based on 16 rRNA an–DNA-DNA hybridization	Cadillo-Quiroz et al. (2014)
Methanobacterium lacus sp. no v	Methanogens	Acidic soil	Phylogeny based on 16 S rRNA an–DNA-DNA hybridization	Borrel et al. (2012)
Methanobactxidesmaludis SWAN	Methanogens	High temperature	Phylogeny based on 16 S rRNA an–DNA-DNA hybridization	Cadillo-Quiroz et al. (2014)
Methanocella arvoryzae	Methanogens	Paddy soil	Pureculture technique & polyphasic taxonomy	Sakai et al. (2010)
Methanocel laconradii	Methanogen	Paddy soil	Pureculture technique & polyphasic taxonomy	Lu and Lu (2012)
Methanoculleus chikugoensis	Methanogenic archaea	Paddy soil	Culture dependent technique	Dianou et al. (2001)
Methanosarcinaceae	Methanogens	Paddy soil	Metagenomic approach	Lueders et al. (2001)
Methylarcula marina VKMB-2159T	Methanotrophs	Marine	Culture dependent technique	Dianou and Adachi (1999)
Methylarcula terricola VKMB-21 60T	Methanotrophs	Marine	Culture dependent technique	Dianou and Adachi (1999)
Methylocella silvestris	Methanotrophs	Acidic forest soil	Culturomics	Dunfield et al. (2003)
Methylomonas EM–L 16-1	Methanotrophs	Marine	Floating filter culture technique	Nguyen et al. (2017)
Methylomonas lineage	Methanotrophs	Marine	Culturomics	Holmes et al. (1999)
Methyloomonus pelugica	Methanotrophs	Marine	Culturomics	Sieburth et al. (1987)
Methylophaga AH 1	Methanotrophs	Marine	Culture dependent technique	Howat (2017)
Methylovulum miyakonense	Methanotrophs	Forrest soil	Phylogeny based on 16 rRNA an–DNA-DNA hybridization	Iguchi et al. (2011)
Novimethylophi luskurashikiensis	Methanotrophs	Paddy soil	Culture dependent technique	Lv et al. (2018)

(Wang et al. 2023b). Additionally, a relatively small cavity in the active site restricts the substrate specificity of pMMO and leads to preferential oxidation of the substrate (R)-alcohols (Pham et al. 2023). In contrast to the comprehensive exploration of methane (C1) metabolism, the breakdown of gaseous alkanes ranging from C2 to C5 among methanotrophs has received limited attention (as depicted in Fig. 5). A noteworthy case lies with the type II methanotroph, *Methylocella silvestris*, which exhibits the capability to utilize both methane and propane as sources of carbon and energy. This distinct trait marks *M. silvestris* as the initial methanotroph known to utilize a short-chain alkane instead of methane. Furthermore, *M. silvestris* is equipped to metabolize ethane through oxidation processes.

The presence of isocitrate lyase and malate synthase explains its ability to thrive on ethane and its metabolic intermediates ethanol and acetate (Bordel et al. 2019). In addition to ethane assimilation, propane assimilation pathways have also been observed in *M. silvestris* (Bordel et al. 2019) and *Methylacidiphilum* sp. IT6 (Awala et al. 2021). Two additional pathways are involved in the degradation of propane: the first converts propane into 1-propanol and then *methylmalonyl*-CoA, whereas the second converts propane into 2-propanol, which is subsequently oxidized to acetone, acetol, and lactate, which can be further converted into pyruvate by putative lactate dehydrogenase (Fig. 5) (Le and Lee 2023).

While cultivating *M. silvestris* on propane, it was observed that both 2-propanol and acetone accumulated in the culture medium. This suggests that the transformation of acetone into acetol could be hindered by kinetic barriers. Intriguingly, during the growth of 2-propanol in *Methylacidiphilum* sp. IT6, a gene cluster responsible for the conversion of 2-propanol to pyruvate via acetol exhibited increased expression. Notably, this cluster encompasses one of the three genomic operons related to pmoA, which generates pMMO. Interestingly, the encoded enzyme PMO3 within this cluster facilities oxidation of acetone to acetol as reported by Awala et al. (2021).

In conclusion, these discoveries shed light on the adaptable metabolic capacities of facultative methanotrophs, showcasing their ability to utilize a wide range of substrates. In the realm of metabolic engineering, given the inherent limitations of carbon flux connected to C1

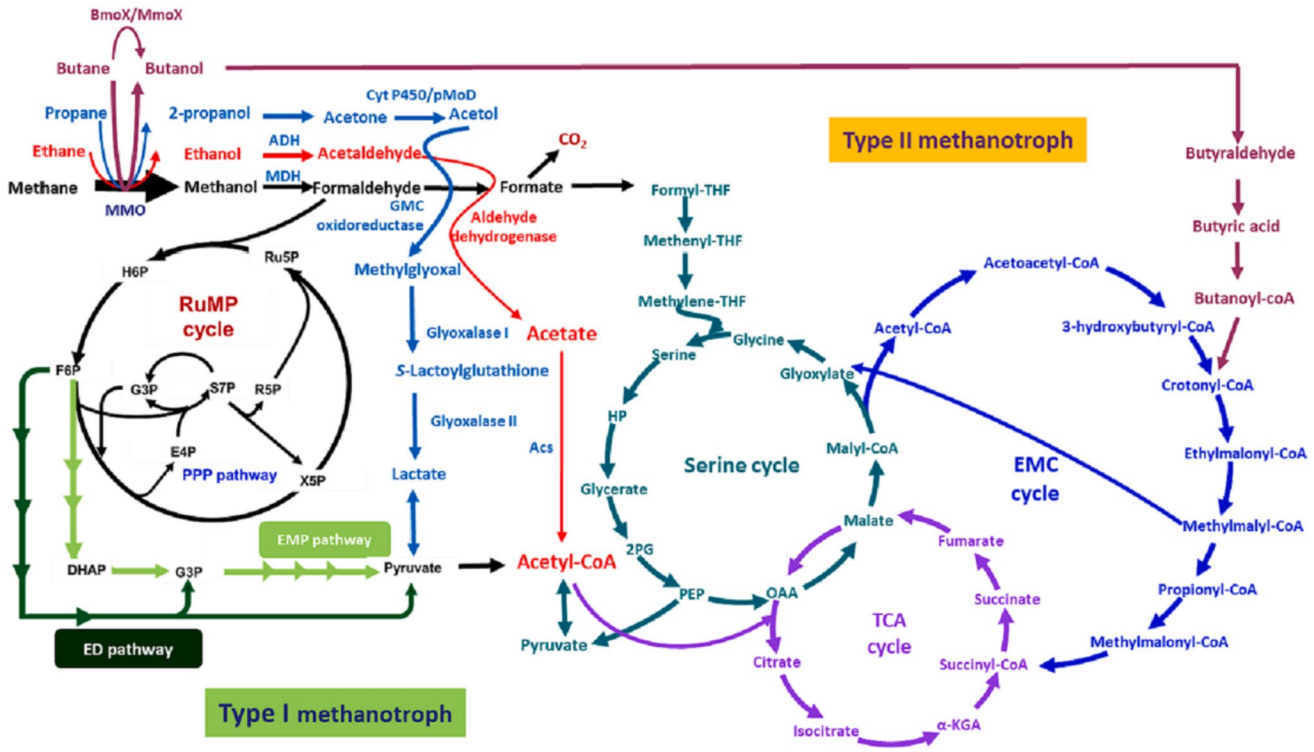

Fig. 5 Methanotrophic bacteria inherently employ various multicarbon sources. Methane, ethane, propane, and butane are subject to oxidation through methane monooxygenase. These oxidation pathways are subsequently transformed through multiple enzymatic steps into central metabolic intermediates. Specifically, methane oxidation is represented by a black line, ethane oxidation by a red line, propane oxidation by a blue line, and the conversion of butane leads to crotonyl-CoA, depicted as a rubine-colored line. Xu5P, xylulose 5-phosphate; Ru5P, ribulose-5-phosphate; S7P, sedoheptulose7-phosphate; G3P, glyceraldehyde 3-phosphate; E4P, erythrose 4-phosphate; F6P, fructose 6 phosphate; H6P, 3-hexulose-6-phosphate; R5P, ribose 5-phosphate; HP, hydroxypyruvate; DHAP, dihydroxyacetone phosphate; MDH, methanol dehydrogenase; ADH, alcohol dehydrogenase (Le and Lee 2023)

compounds, the establishment of facultative growth on co-substrates emerges as a potentially advantageous strategy. This approach could potentially amplify carbon flux directed towards the biosynthesis pathway of desired products within methanotrophic bacteria.

The understanding of methane oxidation mechanisms and the rational engineering of metabolic processes have been challenging due to the exclusive reliance on methane and methanol as carbon and energy sources by obligate gammaproteobacterial methanotrophs. To address the gaps in our foundational comprehension of C1 metabolism and to enhance methanotrophic capabilities for methane conversion, a potential strategy involves merging methanotrophic and heterotrophic metabolic pathways through the construction of synthetic multi-carbon utilization pathways. This approach is especially significant for biorefineries, where cost-effective and abundant methane along with renewable carbon sources can be employed to generate value-added products. Given that obligate methanotrophic bacteria inherently lack the capacity to utilize multi-carbon substrates, the creation of synthetic multi-carbon utilization pathways within host strains becomes essential. This review discusses three multi-carbon utilization pathways present in type I methanotrophs, specifically *M. alcaliphilum* 20Z, as depicted in Fig. 6.

Glycerol-utilizing pathway

One of the drawbacks of methanotrophic bacteria is the requirement of unbalanced reducing equivalents by MMO during the oxidation of methane, which leads to low cell yield of methanotrophs on methane, as well as a lack of reducing equivalents from methane to the formation of reduced products (Pham et al. 2023). Consequently, to bolster cell growth, amplify carbon flow, and increase the availability of reducing agents for producing reduced products, it becomes imperative for these bacteria to engage in the simultaneous metabolism of an additional reduced substrate alongside methane. Glycerol, a by-product of the diesel industry, has been identified as a promising co-substrate due to its widespread availability, notable degree of reduction, and cost-effectiveness (Le and Lee 2023).

To establish a synthetic glycerol-utilizing pathway, three enzymes from *E. coli* including glycerol transporter (*glpF*), membrane-binding FAD^+ dependent glycerol-3 phosphate

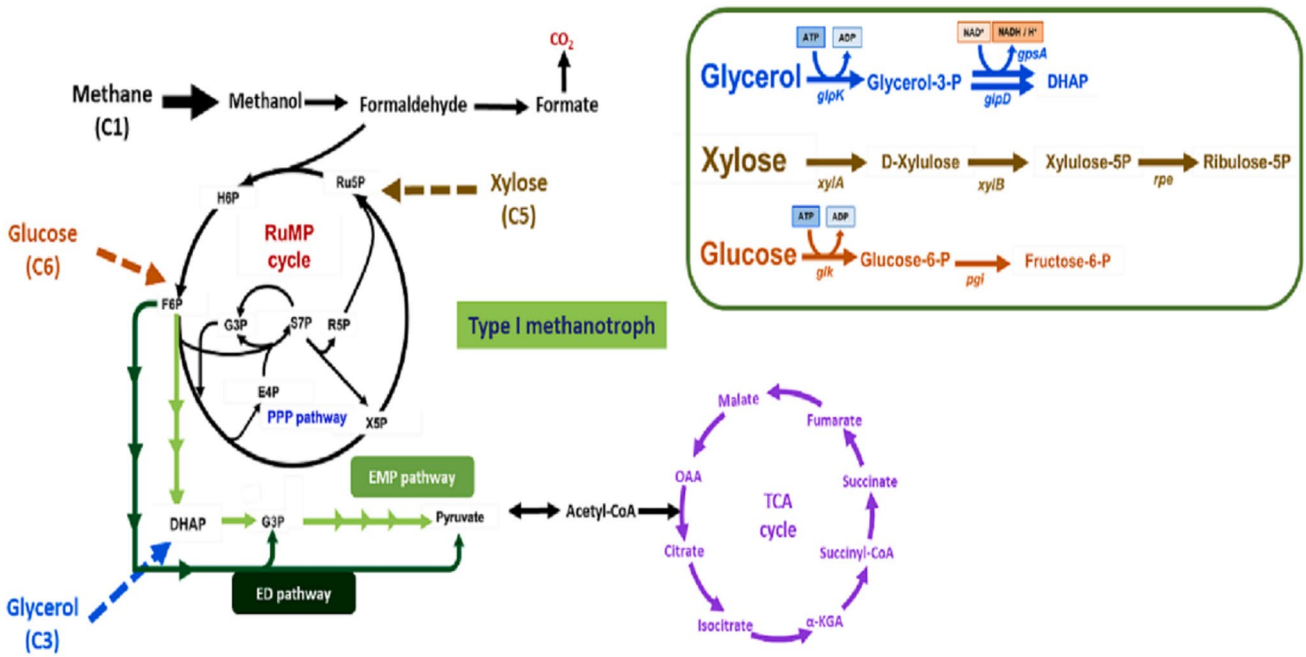

Fig. 6 The synthetic pathways for utilizing multi-carbon compounds were reconstructed within an obligate type I methanotroph. To achieve this, relevant genes associated with each pathway were sourced from various bacteria and subsequently combined. The integration was accomplished by either inserting these genes into the pAW89 expression vector or incorporating them into the genome. Notably, these genes were placed under the regulation of the pTac promoter. These genes collectively contribute to the conversion of various compounds such as Xu5P (xylulose 5-phosphate), Ru5P (ribulose-5-phosphate), S7P (sedoheptulose-7-phosphate), G3P (glyceraldehyde 3-phosphate), E4P (erythrose 4-phosphate), F6P (fructose 6 phosphate), H6P (3-hexulose-6-phosphate), R5P (ribose 5-phosphate), HP (hydroxypyruvate), DHAP (dihydroxyacetone phosphate), MDH (methanol dehydrogenase), and ADH (alcohol dehydrogenase) (Le and Lee 2023)

dehydrogenase (*glpD*), glycerol kinase (*glpK*), and soluble NAD^+ dependent glycerol-3 phosphate dehydrogenase (*gpsA*) from *M. alcaliphilum* 20Z were overexpressed (Le et al. 2021).

Glycerol undergoes a two-step conversion into DHAP (dihydroxyacetone phosphate), subsequently entering the metabolic network (Rozova et al. 2021). It bifurcates towards ribulose monophosphate (RuMP) activity through gluconeogenic flux and towards the TCA cycle through glycolytic flux, facilitated by the activity of fructose-bisphosphate aldolase (*fbaA*) and triosephosphate isomerase (*tpi*), respectively. Through the overexpression of these four genes, noticeable cell growth was observed on glycerol alone, resulting in a final OD600 (optical density at 600 nm) of 1.4 when using 0.1% (v/v) glycerol. However, the genetically engineered strain encountered growth challenges in the presence of higher glycerol concentrations, specifically 0.5% (v/v) and 1% (v/v) (Tyne et al. 2023). This was attributed to the hypothesis that elevated glycerol concentrations might significantly influence osmotic potential, consequently hindering growth (Pham et al. 2023).

Xylose utilizing pathway

In *Methylomicrobium alcaliphilum* 20Z, the primary metabolic pathway is a combination of C1 and five-carbon (C5) sugars (Le and Lee 2023; Nguyen and Lee 2021). Pentose substrates can be directly converted into intermediates of the RuMP cycle, activate the non-oxidative component of the pentose phosphate pathway (PPP) within the RuMP cycle, and support cell growth, as highlighted by Nguyen and Lee (2021). Xylose, a frequently encountered C5 sugar in lignocellulosic biomass, holds significant potential as a versatile multi-carbon feedstock. *Methylomicrobium alcaliphilum* 20Z has demonstrated its ability to assimilate xylose as its sole carbon source through a strategic approach involving the integration of xylose metabolism pathways from *Escherichia coli*, in combination with its native non-oxidative RuMP cycle.

In *E. coli*, the enzyme xylose isomerase (encoded by xylA) converts xylose into d-xylulose. Subsequently, d-xylulose is phosphorylated by xylulokinase (encoded by xylB) to generate xylulose-5-phosphate (Xu5P). The conversion of Xu5P to ribulose-5-phosphate is then facilitated by ribulose-phosphate 3-epimerase. A portion of this ribulose-5-phosphate is incorporated into the non-oxidative PPP. To enable stable xylose utilization in *Methylomicrobium alcaliphilum* 20Z, genetic engineering efforts involved chromosomally integrating the *xylA* and *xylB* genes from *E. coli*, along with the rpe gene from *M. alcaliphilum* 20Z, under the control of the Ptac promoter. This engineering effort resulted in the development of a strain capable of efficiently utilizing xylose as its sole carbon source, as evidenced by observable growth under these conditions.

Glucose utilizing pathway

The enzyme responsible for phosphorylating glucose, ATP-glucokinase (*glk*), has been identified in type I methanotrophs (Zhou et al. 2020). Notably, there are no known methanotrophs capable of utilizing glucose as a carbon source for their growth. Moreover, genome analysis has revealed that some type I methanotrophs may harbor putative gluconate kinase (*GntK*) and glucose-1-dehydrogenase (*gdh*) enzymes, the functions of which remain currently unknown (Rozova et al. 2021). Both gluconate kinase and glucose dehydrogenase could potentially have associations with the Entner-Doudoroff pathway, glycolysis, and the oxidative PPP (Tyne et al. 2023). Double mutants (*gdh—/glk—)* or (*gntk—/glk—)* exhibited distinct phenotypes compared to the wild-type strain of *Methylomicrobium alcaliphilum* when grown on methane. These mutants displayed higher accumulations of glucose-trehalose and lower glycogen storage (Rozova et al. 2021). Despite the presence of redundant sugar metabolism pathways in obligate methanotrophs, the precise reasons for their inability to utilize glucose as a carbon and energy source for growth remain unclear. An intriguing observation is the absence of a phosphotransferase system for glucose transport in type I methanotrophs, which may hinder the uptake of glucose from the surrounding medium into the cells.

Remarkably, Pham et al. (2023) demonstrated the engineered growth of *Methylomicrobium alcaliphilum* 20Z on glucose through the overexpression of three target genes: glucose-facilitated diffusion protein (*glf*) from *Zymomonas mobilis*, native glucokinase (*glk*), and phosphoglucose isomerase (*pgi*) from *E. coli*. In the context of glucose metabolism, it is presumed that cells utilize a heterologous transporter called glf to facilitate glucose uptake from the surrounding medium. Subsequently, glucose is converted into glucose 6-phosphate and fructose 6-phosphate through the actions of glucokinase (*glk*) and phosphoglucose isomerase (*pgi*), respectively.

Molecular biology of sMMO and pMMO

The genes responsible for encoding sMMO from various methanotrophic organisms have been successfully cloned and sequenced. The most extensively studied are those from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (Hwang and Lee 2023). sMMO genes are organized in clusters on the chromosomes of these

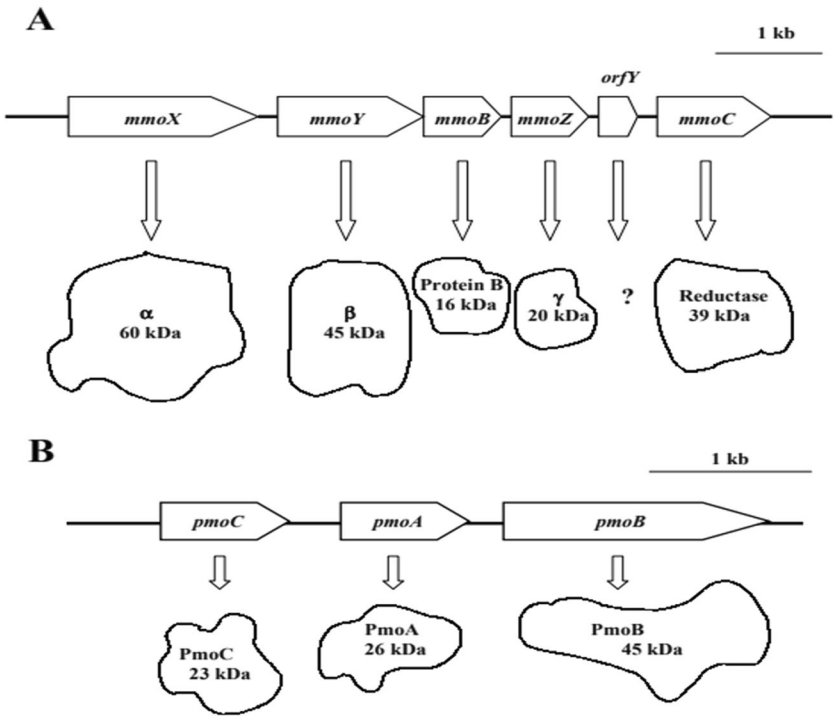

Fig. 7 The soluble methane monooxygenase (**A**) and particulate methane monooxygenase (**B**) gene clusters of methane-oxidizing bacteria (Farhan Ul Haque et al. 2020)

methanotrophic organisms (as shown in Fig. 7A,B). Specifically, within this cluster, *mmoX*, *mmoY*, and *mmoZ* are responsible for encoding the α-, β-, and γ-subunits, respectively, of the hydroxylase. Additionally, *mmoB* and *mmoC* are the genes that encode protein B and the reductase component, respectively.

Interestingly, *mmoB* lies between *mmoY* and *mmoZ*; an ORF of unknown function, designated *orfY*, with a coding capacity of 12 kDa, lies between *mmoZ* and *mmoC* in all genes clusters analyzed to date (Guo et al. 2022). The genes responsible for coding sMMO exhibit a high degree of conservation across all studied methanotrophic genera. The nucleotide sequences of these genes share identities ranging from 55 to 94%, while the corresponding amino acid sequences exhibit similarities ranging from 47 to 96%.

The genes encoding pMMO from *Methylococcus capsulatus* (Bath) have been cloned and sequenced (Dawson et al. 2023) and are clustered on the chromosome in the order *pmoCAB* (Fig. 7B). There are two virtually identical copies of these genes (13 bp changes over 3183 bp of *pmoCAB*) present in the genome of *Methylococcus capsulatus* (Bath) and a third copy of *pmoC* has also been identified (Bo et al. 2023). This is very similar to the analogous system in nitrifiers that also contain two copies of genes encoding ammonia monooxygenase, *amoCAB*, and a third *amoC* gene (Eltayb et al. 2023).

Comparison of *pmo* and *amo* genes from methanotrophs and nitrifiers suggests that the pMMO and AMO may be evolutionarily related (Samanta et al. 2022). The presence of multiple copies of these genes in such bacteria raises questions about their functional significance. In the case of *Nitrosomonas europaea*, for example, the reasons for having multiple copies of the *amoA* gene are not yet fully understood. An interesting observation is that when an insertion mutant with a deficiency in one copy of the *amoA* gene was created, it exhibited slower growth compared to the wild-type strain. Surprisingly, a mutant with a defect in the second copy of the amoA gene showed normal growth. This suggests that while some copies of the gene might be essential for optimal growth, others could be dispensable or have redundant functions.

Singh et al. (2023) successfully developed chromosomal insertion mutants in all seven pmo genes found in *Methylococcus capsulatus* (Bath). Interestingly, except for the singular third copy of the *pmoC* gene, which did not yield any null mutants, the mutants from the other genes displayed growth when supplied with methane. This suggests a functional equivalence between the two sets of genes. Specifically, the mutants related to the first copy demonstrated approximately two-thirds of the methane oxidation activity seen in the wild-type strain, while the mutants related to the second copy exhibited roughly one-third of the activity observed in the wild-type strain. No double null mutants defective in both copies of pmoCAB were obtained which suggests that the cells require pMMO for normal growth (Zhu et al. 2022).

The complete *pMMO* gene clusters from two additional methanotrophic genera, *Methylosinus trichosporium* OB3b and *Methylocystis* sp. strain M, have been recently cloned and sequenced. Similar to other studied methanotrophs, they also possess two copies of the *pmoCAB* genes (as outlined

in Antony et al. 2023). Comparative examination of the derived polypeptide sequences of pMMO and AMO (ammonia monooxygenase) underscores the resemblances between these two enzyme systems and highlights the conserved nature of their sequences. These sequences exhibit identities ranging from 42 to 87% and similarities spanning from 58 to 95% at the amino acid level. *PmoC* and *PmoA* are anticipated to be highly hydrophobic, consisting primarily of putative transmembrane-spanning helices, while *PmoB* contains only two presumed transmembrane regions. The extensive dataset of pMMO gene sequences from various methanotrophs offers the opportunity to utilize *pmo* as a "functional gene probe" in molecular ecology studies, enabling investigations into the diversity of methanotrophs within natural environments. This utilization has been recently explored and reviewed in publications such as Tentori et al. (2022) and Tyne et al. (2023).

Research needs and future direction

Currently, no bacterial strain has demonstrated efficient methane assimilation for both cell growth and chemical production. The challenge lies in achieving stable folding of MMO, with the primary obstacle being the control of DNA-protein crosslinking by formaldehyde during methanol oxidation (Cheng et al. 2022). The development of robust synthetic methanotrophs for targeted product production demands significant efforts in engineering techniques and the study of evolutionary mechanisms, as emphasized by Whiddon et al. (2019). To enhance the efficiency of pMMO in different organisms for methane incorporation, it is crucial to understand its native environment and the specific sites where methane binds. These insights would bridge a substantial knowledge gap for researchers.

Despite recent advancements in methanogenesis and methanotrophy research, particularly the importance of soils like paddy soils in the methane cycle, further exploration is essential to deepen our understanding of these mechanisms and develop innovative eco-friendly methods. Additionally, a more comprehensive grasp of the contributions of various ecosystems to the global methane balance is needed. Several research areas warrant further investigation, including extensive field studies in rice paddies, exploration of novel methane production processes in aerobic conditions (such as non-microbial methanogenic chemical processes and methane production by terrestrial plants), utilization of advanced molecular biology techniques to study high-affinity methanotrophs, and comprehension of anaerobic oxidation of methane (AOM). These endeavors can establish a theoretical framework and a scientific foundation for striving towards "carbon neutrality" within soil ecosystems.

Author contributions FA wrote the original draft, and ML revised the manuscript. All authors read and approved the manuscript.

Data availability Data are available upon request to corresponding author.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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