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Review

Advances in synthetic methanotrophy for sustainable C1 bioconversion

Claudia Tahiraj[1,6], Lucas Hille[2,6], Karen Brunsbach[1,6], Michael Macgregor-Fairlie[2], Valentin Steier[3], Steffen N. Lindner[2], Rahul Banerjee[4], and Lars Lauterbach[1,5,*]

Methane (CH_4) represents an abundant source of carbon and energy with significant potential for sustainable biotechnological processes. For efficient bioconversion of CH_4 into value-added products, synthetic methanotrophy has emerged as a promising strategy, enabling the rational design of engineered microbial systems. In this review, we highlight recent advances in CH_4-based bioprocesses, covering the metabolic design of synthetic methanotrophs and optimization of bioreactor systems adapted for gas fermentation. A comparative analysis of key CH_4-converting enzymes is provided, with particular emphasis on soluble methane monooxygenase and its heterologous production in industrial chassis. Recent progress in modular one-carbon (C1)-pathway engineering accelerates enzyme optimization and highlights synthetic methylotrophy as a prerequisite for robust synthetic methanotrophy. Collectively, these advances establish a foundation for scalable, efficient, and sustainable CH_4-based biotechnological processes.

Highlights

Functional production of soluble methane monooxygenase (sMMO) in *Escherichia coli*, supported by chaperonins and maturases, and the first methane (CH_4)-to-methanol conversion by a truncated mini-sMMO variant represent major advances toward synthetic methanotrophy in industrial hosts.

Natural CH_4-oxidizing enzymes [sMMO, particulate methane monooxygenase (pMMO), methyl-coenzyme M reductase (MCR)] and synthetic *de novo* monooxygenases (mini-sMMO) are engineered as complementary biocatalysts for CH_4 oxidation in synthetic methanotrophy.

Miniaturized, online-monitored CH_4 fermentation platforms enable systematic optimization of gas transfer, pressure control, and safety management, enhancing process scale-up.

Fully synthetic methylotrophy has been achieved in model organisms, confirming the feasibility of complete synthetic one-carbon (C1) assimilation as an alternative to natural pathways. Development of methylotrophic biosensor strains in traditional hosts provides a powerful tool for growth-coupled engineering, while also offering a genetically tractable framework for integrating CH_4 oxidation modules.

The convergence of synthetic methylotrophy, engineered CH_4-oxidizing enzymes, and gas bioprocess technologies establishes a blueprint for scalable, circular, and carbon-neutral CH_4-to-chemical conversion.

Methane as a carbon and energy source for bioproduction

Methane (CH_4), the most reduced C1 moiety on Earth, is a highly promising and underused carbon source for microbial bioprocesses in industrial biotechnology [1]. It offers potential advantages over conventional feedstocks, such as glucose and molasses, because it does not compete with food production, thereby addressing a key challenge in sustainable resource allocation. As a gas, CH_4 enables scalable **gas fermentation** (see Glossary) without having to consider the addition of liquid nutrients, unlike carbohydrate-based feedstocks. This simplifies supply chains, eliminating the need for costly carbohydrate inputs by using methanotrophic bacteria capable of utilizing CH_4 as both a carbon and energy source [2].

In many regions, CH_4 derived from fossil resources remains the most economically viable carbon source (e.g., shale gas in the USA), creating opportunities to produce single cell protein (SCP), polyhydroxyalkanoates (PHA), and bulk chemicals using microbial bioprocesses. However, decentralized biotechnological solutions are increasingly needed, because CH_4 is often simply flared or vented due to economic constraints, leading to its conversion back into CO_2 without valorization. Companies, such as STRING Bio, Industrial Microbes, and Mango Materials, have already demonstrated technological readiness for harnessing methanotrophs in biotechnological applications, underscoring the feasibility of CH_4-based processes.

Central to this potential is the unique metabolic capability of aerobic methanotrophs to activate CH_4. These organisms can cleave the strong C–H bond of CH_4 and convert it into methanol under ambient conditions as the initial step of their metabolism, mediated by methane monooxygenases (MMOs) [3,4]. The methane-to-methanol pathway also provides an efficient platform for chemical synthesis and aligns well with existing liquid transportation infrastructure,

[1]RWTH Aachen University, Institute of Applied Microbiology, Aachen, Germany
[2]Charité Universitätsmedizin Berlin, Department of Biochemistry, Berlin, Germany

enhancing practical feasibility for integration into **industrial biorefineries** [5,6]. Methanol acts as a versatile intermediate for producing a range of industrially relevant products, including formaldehyde, organic acids, and fine chemicals. From a metabolic engineering perspective, methanol serves a central role not only for natural methanotrophs, but also for synthetic approaches that seek to engineer CH_4 utilization in non-native hosts. Therefore, beyond its industrial value, methanol serves as a metabolic gateway, offering a strategic entry point for extending methylotrophic capabilities toward full synthetic methanotrophy in **industrial chassis**. As industries aim to transition toward more sustainable practices, utilizing CH_4 represents a transformative shift that reduces costs, minimizes environmental impact, and opens new avenues for bio-based production [5]. Continued advances in gas fermentation technologies, sustainable CH_4 production, and metabolic engineering of methanotrophs will further strengthen the role of CH_4 as a key feedstock in next-generation industrial biotechnology. Herein, we discuss the current state-of-the-art of **synthetic methanotrophy** and how its potential in industrial settings can be harnessed in relation to sustainable routes of CH_4 synthesis, thus providing a holistic perspective on circular CH_4 utilization.

Sustainable routes of methane synthesis

With the emergence of anthropogenic climate change, CH_4 emissions are increasingly recognized as a major environmental concern. As a readily accessible hydrocarbon, CH_4 arises from agriculture, natural, and industrial processes, and is released during the extraction of fossil fuels (Figure 1). Although this latter source of CH_4 remains abundant, the emergence of anthropogenic climate change means that CH_4 emissions are increasingly recognized as a major environmental concern. Therefore, it is vital to analyze and understand natural and engineered biological routes of synthesis while also identifying emerging work in synthetic methanotrophy, highlighting the possibility of repurposing microbial platforms for sustainable CH_4 conversion. Despite associated challenges, there also lies a great opportunity and, therein, the potential to shift toward a

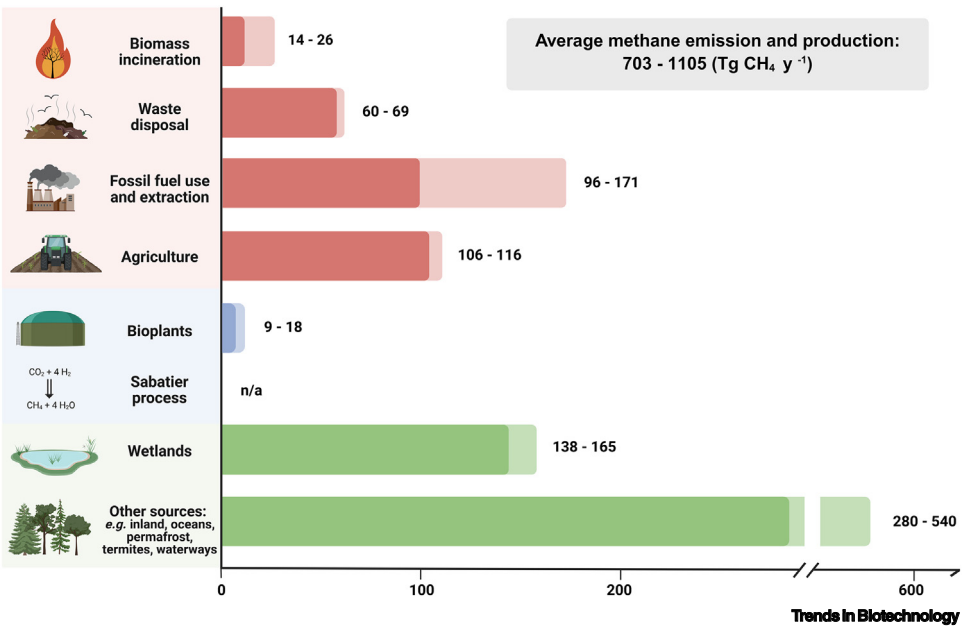

Figure 1. Sources of methane (CH_4) emission and production with approximate metrics. Red bars indicate anthropogenic CH_4 emissions, blue bars represent sustainable CH_4 production processes, and green bars show natural CH_4 sources. Quantitative values are given in teragrams CH_4 per year (Tg CH_4 $year^{-1}$) where available; n/a indicates that no reliable global estimate exists.

[3]RWTH Aachen University, Aachener Verfahrenstechnik – Chair of Biochemical Engineering, Aachen, Germany
[4]Auburn University, Department of Chemistry and Biochemistry, Auburn, AL, USA
[5]Fraunhofer-Institut für Molekularbiologie und Angewandte Ökologie IME, Aachen, Germany
[6]These authors contributed equally

*Correspondence:
lars.lauterbach@rwth-aachen.de
(L. Lauterbach).

sustainable and circular economy that not only mitigates emissions, but also utilizes greenhouse gases, such as CH_4, as valuable resources [7]. While CH_4 from fossil resources remains abundant, reliance on these sources undermines the environmental benefits of bio-based production. Sustainable methods to acquire CH_4, such as biogas production and the **Sabatier process**, offer promising alternatives to fossil fuel-based sources and directly support the aim of synthetic methanotrophy, namely the integration into sustainable, circular value chains [8,9].

Biogas production relies on methanogenic archaea that generate CH_4 as a metabolic by-product in anaerobic digesters. The process proceeds through four main steps: hydrolysis, acidogenesis, acetogenesis, and methanogenesis, which can occur either in a single-stage or a more controlled two-stage system [9]. The first three steps convert complex organic substrates into acetic acid, CO_2, and H_2, which are subsequently used by methanogens to produce CH_4. In parallel, the Sabatier process provides a nonbiological route for CH_4 synthesis by catalyzing the reaction of CO_2 and H_2. Since its first description by Paul Sabatier in 1902, numerous iron- and nickel-based catalysts have been developed to enable low-temperature CO_2 reduction, although challenges remain regarding CO_2 selectivity and catalytic efficiency [10].

Initially used for CO and CO_2 removal from H_2 streams, interest in this process resurged during the 1970s oil crisis and is now central to power-to-methane strategies, which will be used to fuel long-term space missions [8,11]. Access to renewable CH_4 through ecological and sustainable methods, such as biogas production and the Sabatier process, enables engineered microbial systems to convert CH_4 into valuable products in a circular and environmentally responsible manner. By integrating these fossil-independent CH_4 sources, industrial applications utilizing synthetic methanotrophs may also help establish economically competitive and sustainable value chains.

Methane-based bioprocesses

Methane-based bioprocesses require a complex understanding of the interplay of different factors hampering the efficiency of CH_4 bioconversion. The most important factors affecting CH_4-based bioprocesses are gas–liquid transfer limitations of CH_4 as well as safety considerations due to possibly explosive gas mixtures (5–17 mol% CH_4 in air) in the **headspace** [12,13]. The latter can be overcome by using nonexplosive gas mixtures or **ATmosphères EXplosibles (ATEX)**-certified bioreactors [14]. Methanotrophic growth on CH_4 requires a tightly controlled gas composition in the headspace of reactor units. Safe gas mixtures contain low CH_4 and O_2 concentrations to guarantee a nonexplosive atmosphere in case of leakage and mixing with ambient air [13]. This is likely accompanied by limitations of gaseous substrates throughout the cultivation process [15]. Even with higher CH_4 concentrations in ATEX-certified settings, the limitations of gas–liquid transfer and the low solubility of CH_4 in water (1.4 mM at 25°C, 1 atm, 100 mol%) typically result in low availability of CH_4 for microorganisms and substrate limitations. Methanotrophs naturally cope with low availability of CH_4 by containing MMOs with high affinity toward CH_4 in the lower micromolar range, ensuring efficient CH_4 uptake [16,17]. In bioprocesses, CH_4 availability can be increased by elevating CH_4 partial pressure to optimize gas transfer or using membrane-based aeration or membrane bioreactors [18,19]. For this, various approaches and bioreactor configurations (airlift reactor, stirred-tank reactor, trickle-bed reactor, membrane reactors, and two-phase reactors) have been evaluated, as reviewed extensively elsewhere [20–22].

While these approaches may alleviate gas transfer limitations at the laboratory scale, they are usually difficult to scale up to production scale due to complexity or cost. In general, pneumatically agitated reactors (bubble column or airlift) are recommended for gas-based production processes due to high gas hold up and economic advantages [23]. Here, it is recommended to follow

Glossary

ATmosphères EXplosibles (ATEX): certification confirming that equipment meets the ATEX safety standards for use in explosive atmospheres of the European Union.
Auxotrophy: inability of an organism to synthesize a particular compound required for its growth, necessitating its external supply.
Biosensor strain: genetically modified organism that produces a detectable signal (e.g., growth) in response to a specific metabolite or environmental condition.
C1 assimilation: biological incorporation of C1 compounds, such as CH_4, methanol, or formate, into biomass or central carbon metabolism.
Carbon yield: fraction of consumed carbon substrate that is converted into the desired product or biomass.
Central carbon metabolism: core metabolic pathways that process carbon sources to generate energy and biosynthetic precursors.
Chaperonin: protein that aids the correct folding of polypeptides into functional 3D structures.
Compound Q: hypervalent $[Fe^{IV}Fe^{IV}]$-oxo species that is a reactive intermediate in oxidative cleavage of the strong C–H bond in CH_4 catalyzed by soluble methane monooxygenases.
Directed evolution: technique that mimics natural selection to evolve proteins or pathways with enhanced or novel functions through iterative mutagenesis and screening.
Energetic efficiency: effectiveness with which cells convert C1 carbon sources (e.g., CH_4, methanol, formate, or CO_2) into usable biochemical energy, such as ATP or reducing power.
Gas fermentation: biotechnological process in which gaseous substrates (e.g., CH_4, CO_2, CO, or H_2) are used by microorganisms as carbon and/or energy sources to produce biomass or value-added chemicals under controlled conditions.
Growth-coupled engineering: method that links cell growth to the production and function of a respective protein or pathway, allowing selective pressure to favor improved variants.
Headspace: gas phase above the liquid culture broth in an enclosed cultivation system (e.g., bioreactor or serum bottle), which serves as a reservoir for gaseous substrates.

a knowledge-based scale-up and scale-down approach that ensures reproducible process performance across different scales, including the use of models for hydrodynamic behavior within the process and metabolic models for the respective methanotroph [24]. For more efficient process development and faster validation of model predictions, parallel miniaturized cultivation systems are required to increase experimental throughput for process development. Serum bottles can be used for this purpose, with gas conversion monitored via pressure sensors; however, these lack gas selectivity and do not maintain a constant gas composition [25]. The use of different gas compositions or anaerobic conditions has also been demonstrated at the microtiter scale, such as in BioLector systems, and other parallel miniaturized bioreactor platforms, offering online monitoring of biomass concentration and other critical bioprocess parameters [26–29]. Methane-based bioprocesses might also be realized in such systems, albeit restricted to nonexplosive gas mixtures [30].

The online measurement of gas transfer rates is crucial to monitor substrate availability, and identify limitations and inhibitions; it also gives insight into the metabolic state of the cultivated organism [31,32]. This principle was successfully adapted to measure the CH_4 transfer rate of *Methylococcus capsulatus* (Bath) in shake flasks [15]. Beyond this example, online monitoring of CH_4 availability and conversion remains a challenge. Off-gas measurement of CH_4 often requires ATEX-certified equipment, while measurements of dissolved CH_4 concentrations have been developed, but are not commercially available for bioprocesses [33–35].

Insights from gas fermentation provide valuable guidance for the technological development of synthetic methanotrophy. Continuous cultivation under a defined and safe CH_4/O_2 atmosphere, as established for native methanotrophs, represents a prerequisite to enable not only growth, but also adaptive optimization of heterologous CH_4-assimilating pathways in synthetic methanotrophic hosts. Thus, strategies to overcome gas–liquid transfer limitations, maintain nonexplosive gas mixtures, and monitor gas transfer rates will be essential for allowing these engineered pathways to function efficiently and to adapt to CH_4 as a substrate. In this way, advances in CH_4-based bioprocesses provide a technological foundation for realizing stable, synthetic CH_4 conversion.

Advances in synthetic methanotrophy

In addition to the challenges associated with CH_4-based gas fermentation, the specific growth requirements of native methanotrophs and limited genomic accessibility pose significant barriers to their broader use [36,37]. Despite genetically engineering native methanotrophs to produce value-added products from CH_4 and ongoing efforts in the development of genetic tools, their industrial applicability remains limited [19,38–43]. As a result, research has increasingly focused on the heterologous production of CH_4-oxidizing enzymes, thus representing a shift in the potential of synthetic methanotrophy in well-established industrial chassis, such as *Escherichia coli*. By implementing CH_4-assimilating pathways in industrially optimized strains of *E. coli*, the range of products that can eventually be produced is significantly broadened, offering an attractive alternative to implementing product biosynthesis pathways in native methanotrophs.

A range of alkane-oxidizing **metalloenzymes** exist that are capable of aerobic or anaerobic oxidation of CH_4 to methanol. Therefore, various key enzymes known to show CH_4-oxidizing activity can be considered as candidates for heterologous production in non-native hosts to eventually establish synthetic methanotrophy. The particulate MMO (pMMO) is a heterotrimeric, membrane-bound enzyme containing copper active sites catalyzing aerobic CH_4 oxidation [44]. Furthermore, the ammonia monooxygenase (AMO) is a close homolog of pMMO that also exhibits aerobic CH_4-oxidizing activity. Notably, AMO displays a broader substrate specificity

Industrial biorefinery: large-scale facility that converts renewable biomass or waste carbon sources into fuels, chemicals, or materials through biotechnological processes.
Industrial chassis: microbial host strain optimized for industrial biotechnology applications, often characterized by robustness, genetic tractability, and high production capacity.
Maturase: protein that assists proper metal incorporation, cofactor integration, biosynthesis, protection, and assembly of subunits required for enzymatic activity.
Metalloenzyme: enzyme that contains one or more metal ions essential for its catalytic activity.
Non-native host: organism equipped to express a particular gene or pathway that does not natively occur in the host organism.
Rational protein engineering: design of improved or novel proteins based on knowledge of their structure and function.
Sabatier process: chemical synthesis process in which CO_2 is catalytically hydrogenated with H_2 to produce CH_4 and water, typically using a nickel-based catalyst under elevated temperature and pressure.
Self-sufficient CYP: cytochrome P450 monooxygenase capable of performing oxidation reactions independently of external redox partners (e.g., ferredoxin or reductase module).
Synthetic methanotrophy: engineering of a non-native host to metabolize CH_4 as a sole carbon and energy source through introduced metabolic pathways.
Synthetic methylotrophy: engineering of non-native hosts to metabolize methanol as a sole carbon and energy source through introduced metabolic pathways.

compared with pMMO [45]. However, both enzymes are membrane associated and form complex multimeric structures, which makes their heterologous production challenging. Furthermore, attempts to heterologously express the pMMO gene cluster in *E. coli* have been reported to cause cellular toxicity [46]. This significantly limits their utility for synthetic pathway engineering in industrial hosts, such as *E. coli*.

In terms of cytosolic enzymes, soluble MMO (sMMO) is a key enzyme involved in aerobic CH_4 oxidation in native methanotrophs. Generally, aerobic CH_4 oxidation requires the input of two electrons to activate O_2, entailing an energy-demanding step. In the sMMO complex, the reductase module MmoC supplies electrons through NADH oxidation, and the sMMO activity is facilitated by the regulatory protein MmoB [47]. Due to its central role, unique properties, and applicability under aerobic conditions, this enzyme remains the key research target among CH_4-oxidizing enzymes.

In addition to canonical MMO systems, cytochrome P450 (CYP) monooxygenases have emerged as promising alternatives. Recently, heme-dependent and **self-sufficient CYP** monooxygenases exhibiting aerobic CH_4-oxidizing activity were identified. For example, when heterologously produced, a self-sufficient CYP monooxygenase from *Caldimonas thermodepolymerans* demonstrated conversion of CH_4 to methanol and subsequently to C3–C4 products in a multienzyme cascade [48]. Self-sufficient CYP enzymes offer a key advantage in that their ferredoxin domain is fused to the monooxygenase, thus obviating the requirement for a ferredoxin transfer partner. This also reduces the need to form multimeric assemblies or complex post-translational maturation, as seen in canonical CYP systems.

In contrast to enzymes catalyzing aerobic CH_4 oxidation, methyl-coenzyme M reductase (MCR) represents a distinct class of enzymes capable of anaerobic CH_4 oxidation, which does not consume reducing equivalents for O_2 activation, making it an energy-efficient alternative. MCR has a central role in the thermodynamically efficient CH_4 metabolism of anaerobic methanotrophic archaea (ANME) and physiologically related methanogens, which either utilize or produce CH_4 via the reversible methanogenesis reaction depending on environmental conditions. MCR contains a nickel-hydrocorphin prosthetic, namely coenzyme F_{430}, for catalyzing the anaerobic oxidation of CH_4, while the underlying reaction mechanism remains poorly understood. Although MCR exemplifies an alternative principle of CH_4 activation distinct from monooxygenase systems, its relevance for synthetic methanotrophy remains largely conceptual rather than practical, since the heterologous production of MCR under anaerobic conditions in traditional hosts, such as *E. coli*, has so far proven intractable. Moreover, its applicability is constrained due to its high sensitivity to O_2 and to the thermodynamically unfavorable nature of anaerobic CH_4 oxidation compared with methanogenesis, which occurs naturally only when coupled to highly favorable electron acceptors, mainly through syntrophic interactions [49].

Recent advances have considerably expanded our understanding of CH_4-oxidizing enzymes, including the investigation of multiple sMMO variants from diverse microbial sources, various CH_4-oxidizing CYP monooxygenases, as well as MCRs. These developments are summarized in Table 1, which compiles and compares reported kinetic parameters of isolated enzymes toward CH_4 conversion.

Soluble methane monooxygenase

The well-studied sMMO operates through a complex reaction cycle involving multiple protein components and intermediates. Central to this cycle is the hydroxylase MMOH, which comprises a dimer of heterotrimers: the [FeFe] active site-containing MmoX ($MMOH_\alpha$), MmoY ($MMOH_\beta$), and MmoZ ($MMOH_\gamma$) [50]. Furthermore, the regulatory protein MmoB and the NADH-dependent

Table 1. Activity datasheet of cytosolic CH_4-converting enzymes

CH_4-converting enzyme	Native organism/origin	Auxiliary systems	Active site/ cofactors	Kinetic parameters of isolated enzymes towards CH_4				Reductant	Refs
				k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	TTN		
Soluble methane monooxygenase									
sMMO	*Methylococcus capsulatus* (Bath)	MmoB, MmoC, MmoD, MmoE, MmoG, MmoR, [2Fe-2S], FAD biosynthesis	Dinuclear iron site (MMOH), [2Fe-2S] (MmoC), FAD (MmoC)	0.2–1.0	3	67 667–333 333	n/a	NADH	[98,99]
	Methylosinus trichosporium OB3b	MmoB, MmoC, MmoD, MmoE, MmoG, MmoR, [2Fe-2S], FAD biosynthesis	Dinuclear iron site (MMOH), [2Fe-2S] (MmoC), FAD (MmoC)	3.7	12	308 000	n/a	NADH	[100]
mini-sMMO sm1	Synthetic construct of engineered sMMO components derived from *M. capsulatus* linked to human apo-ferritin scaffold	FAD biosynthesis	Dinuclear iron site (ΔH_α), FAD (R_{FAD})	0.32	n/a	n/a	n/a	NADH	[63]
Cytochrome P450 monooxygenase									
CYP153A6	*Myobacterium* sp. HXN-1500	Ferredoxin reductase (FrdA6), ferredoxin (FrxA6), heme, [2Fe-2S], FAD biosynthesis	Heme iron-protoporphyrin IX, FAD (FrdA6), [2Fe-2S], FAD (FrxA6)	n/a	n/a	n/a	0.05	NADH	[101]
CYP102A1 (P450BM3)	*Priestia megaterium*	Heme, [2Fe-2S], FAD biosynthesis	Heme iron-protoporphyrin IX, FAD, [2Fe-2S]	n/a	n/a	n/a	4	NADPH	[102]
CYP116B	*Caldimonas thermodepolymerans*	Heme, [2Fe-2S], FAD biosynthesis	Heme iron-protoporphyrin IX, FAD, [2Fe-2S]	0.03	18	1,667	99	NADPH	[48]
Methyl-coenzyme M reductase									
ANME-1 MCR	*Methanothermobacter marburgensis*	F_{430} biosynthesis	Nickel-tetrahydrocorphin (coenzyme F_{430}), CoM-S-S-CoB	n/a	>1 mM	n/a	n/a	n/a	[103]
	Unculturable ANME-1 from black sea mat	F_{430} biosynthesis	Nickel-tetrahydrocorphin (coenzyme F_{430}), CoM-S-S-CoB	n/a	n/a	n/a	n/a	n/a	[104]

reductase MmoC are both associated with the sMMO complex (Figure 2). The regulatory function of MmoB determines the activity of MMOH by modulating the access of substrates and release of products, thereby driving the sMMO reaction cycle [51,52]. The reductase MmoC contains both a

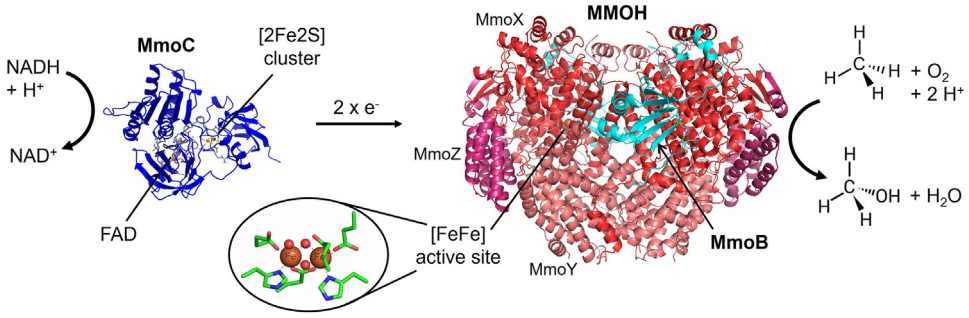

Figure 2. Biocatalytic methane oxidation by soluble methane monooxygenase (sMMO). sMMO consists of three components: hydroxylase (MMOH, red), regulatory protein (MmoB, cyan), and NADH-dependent reductase (MmoC, dark blue). MMOH is a multisubunit protein with a homodimer $(\alpha\beta\gamma)_2$ architecture, comprising MmoX ($MMOH_\alpha$), MmoY ($MMOH_\beta$), and MmoZ ($MMOH_\gamma$). The dinuclear iron [FeFe] active site is contained within MmoX and serves as the catalytic center for methane (CH_4) oxidation. MmoB binds to the canyon region of MMOH, where it regulates substrate access and sMMO activity. The reductase MmoC transfers electrons from NADH to MMOH and contains both a FAD cofactor and a [2Fe2S] cluster, which together mediate electron transfer to the [FeFe] active site. sMMO model of *Methylomonas methanica* MC09 generated with AlphaFold 3.

FAD and a [2Fe2S] cluster. The NADH coenzyme in MmoC serves as an electron donor for the reduction of the catalytic center $[Fe^{III}Fe^{III}]_{ox}$ to $[Fe^{II}Fe^{II}]_{red}$ [47,53]. Once MmoC dissociates, the reduced $MMOH_{red}$:MmoB complex rapidly binds O_2, forming a hypervalent $[Fe^{IV}Fe^{IV}]$-oxo species known as **compound Q** [52–55]. Compound Q enables the proposed radical-based cleavage of the strong C–H bond in CH_4 (104 kcal mol^{-1}) [56].

Since MmoB and MmoC compete for the same binding site on MMOH with similar affinities, the precise timing of complex formation ensures the efficient completion of the sMMO catalytic cycle by preventing reduced MmoC from quenching the intermediate Q oxidant [57]. The same sMMO-binding site can be recognized by MmoD, suggesting a dual inhibitory effect by both competing with the enhancer MmoB and suppressing the formation of catalytic intermediates [58,59]. MmoD induces distinct conformational changes in MMOH, including displacement of the MmoY N-terminal helix and structural rearrangements disrupting the geometry of the [FeFe] active site [58]. Although its physiological role remains debated, MmoD is thought to act as a **maturase** by stabilizing the immature sMMO complex and protecting it from proteolytic degradation during assembly, an aspect that is particularly important for heterologous production for synthetic methanotrophy [58,60].

Heterologous production of sMMO in **non-native hosts** was first accomplished in *Pseudomonas putida*, *Agrobacterium tumefaciens*, and *Rhizobium meliloti*, specifically showing activity in trichloroethylene decomposition, but none of these systems were reported to oxidize CH_4 [61,62]. Conversely, despite substantial efforts from various research groups, active sMMO production in *E. coli* has remained elusive for decades. However, significant breakthroughs were reported by demonstrating the successful heterologous production of active sMMO in *E. coli* [60,63,64].

The first demonstration of active sMMO, heterologously produced in *E. coli*, showed specific activity of the purified sMMO complex with the model substrate nitrobenzene (71 ± 1 mU mg^{-1}), approaching the activity of native sMMO from *Methylosinus trichosporium* OB3b (150 mU mg^{-1}) [64]. Yet, a limitation in heterologous sMMO production has been the tendency of the hydroxylase subunits to form insoluble aggregates, representing a major bottleneck for obtaining active enzyme. The challenge to produce a correctly folded and mature hydroxylase MMOH of sMMO has been overcome by the co-production of **chaperonins**, such as the heat-shock chaperonins

GroESL and the structurally homologous GroES:MmoG complex [60,64]. The auxiliary protein MmoG, a putative chaperonin in methanotrophs, exhibits high structural homology to the GroEL chaperonin (Cpn60), but notably lacks a GroES component attributed to the small subunit of the GroESL complex [64,65]. The similarity of MmoG to GroEL and experimental evidence indicates that MmoG acts as chaperonin for MmoX [60,66].

Furthermore, an engineered miniature sMMO (mini-sMMO) variant marked the first demonstration of CH_4 oxidation in *E. coli*. *In vitro* CH_4 oxidation was confirmed by measuring methanol formation in a CH_4-purged reaction buffer containing purified sm1, yielding an estimated turnover frequency of 0.32 s^{-1} over the initial 15 min of reaction. In pH-stat fed-batch conditions using whole cells, sm1-producing *E. coli* converted CH_4 with a recoverable methanol productivity of 0.11 g L^{-1} h^{-1} [63]. Nonetheless, this system represents a highly engineered synthetic construct derived from sMMO and is not directly comparable to canonical sMMOs, particularly because the reported methanol titer was obtained under high cell density fermentation conditions that do not reflect the physiological state of methanotrophic growth.

More recently, *in vivo* whole-cell activity assays using *E. coli* strains heterologously producing sMMO, reported the hydroxylation of the model substrate naphthalene, detected qualitatively by high-performance liquid chromatography (HPLC). Nevertheless, isolated sMMO could only be obtained after the separate purification and renaturation of hydroxylase components from inclusion bodies, and neither *in vitro* activity nor CH_4 oxidation was demonstrated in this system [60].

Taken together, these findings highlight considerable advances in heterologous sMMO production while also underscoring persistent limitations. The inherent accumulation of insoluble sMMO forming inclusion bodies, along with its complex multisubunit structure and reliance on cellular cofactors and chaperonins, poses major challenges for stable, functional production in heterologous hosts, underscoring the need for further molecular and bioprocess optimization.

The production of sMMO involves a highly complex regulatory and structural system, and the broad substrate scope of the enzyme further underscores its functional versatility in organisms that depend exclusively on CH_4 oxidation. Although sMMO is capable of oxidizing a variety of nonpolar aliphatic and aromatic compounds, only CH_4 serves as its metabolically relevant substrate, suggesting that sMMO operates through a mechanism to selectively favor the oxidation of CH_4, despite it being the most stable and nondescript substrate among all potential substrates [3,67]. Moreover, sMMO must prevent the overoxidation of methanol, a challenge that has hindered the development of small-molecule catalysts for hydrocarbon oxidation [68]. The precise timing of substrate entry, catalysis, and product release is tightly regulated by access tunnels, internal cavities, and other structural strategies (Box 1) [69]. The efforts in protein engineering of sMMO for elucidating the reaction cycle, exploring the substrate cavity, and enhancing protein solubility are summarized in a comprehensive mutant library featuring designed variants (Table 2).

Development of synthetic methylotrophs as the foundation of synthetic methanotrophy

Natural and **synthetic methylotrophy** represent an essential foundation for engineering synthetic methanotrophy, since the expansion from a methylotrophic to a methanotrophic metabolism requires only a single additional enzymatic reaction, the oxidation of CH_4 catalyzed by sMMO. Methanol-dependent strains can thereby serve as **biosensor strains** for the functional assembly and activity of sMMO and be used simultaneously as a tool to accelerate the adaptation of synthetic methanotrophic pathways in non-native hosts. Consequently, advances in engineering methylotrophy is a key prerequisite toward establishing synthetic methanotrophy [70–73].

Box 1. Substrate tunnels and cavities of sMMO

In sMMO, three hydrophobic pockets (cavities 1, 2, and 3, Figure I) extend from the active site to the protein surface, along with a pore connecting cavity 1 directly to the surface [50,69,105]. These cavities are suggested to provide a route for CH_4 and O_2 entry due to the presence of substrate and product molecules in cavities 1 and 2 [106,107]. Specifically, residues Phe188 and Leu110 form a gate closed in oxidized MMOH from *Methylococcus capsulatus* (Bath), controlling access to the [FeFe] site from cavities 2 and 3. Upon binding of MmoB to MMOH, residue Phe188 shifts, connecting the two cavities and supporting the gating model [67,108]. Electron density maps of the MMOH:MmoB complex suggest that an unmodeled substrate molecule perturbs the gate. However, kinetic data do not support CH_4 accessing the [FeFe] site via the 35-40 Å chain of cavities. The linear decay rate of intermediate Q with substrate concentration contradicts the notion of cavities filling with CH_4 before catalysis, implying that alternative access routes are involved [108].

Two possible alternatives for substrate access to the active site have been proposed. First, direct entry might be available through the pore region, although MmoB binding blocks access to the [FeFe] center [109,110]. Second, a recently identified narrow tunnel, designated W308-tunnel 1, was discovered using a probe with a smaller solvent radius. This tunnel is gated by residues Pro215 and Trp308 and is lined with conserved hydrophobic residues. The W308-tunnel 1 is occluded in oxidized MMOH, however, it becomes accessible in the reduced MMOH:MmoB complex from *Methylosinus trichosporium* OB3b [69,110]. Binding of MmoB organizes a dome of hydrophobic residues at the entrance, proposed to facilitate O_2 entry. As demonstrated by mutagenesis studies, the tunnel is adjacent to several MmoB residues critical for catalysis. For instance, replacing the MmoB residue Val41, located at the tunnel entry to MmoX, with arginine or other bulky residues almost completely abrogated enzymatic activity [110].

While both the chain of cavities and W308-tunnel 1 have been proposed as access routes for CH_4 and O_2, recent work suggests another related path, termed W308-tunnel 2, is presumably responsible for CH_4 access. This alternative path is widened in complex between MMOH and a double mutant $MmoB^{S109A/T111A}$, which corresponds with this MmoB variant exhibiting increased reactivity rates with larger substrates [57]. The application of targeted derivatives enabled detailed investigation of structural and functional interplay between individual subunits and permitting controlled modulation of substrate specificity.

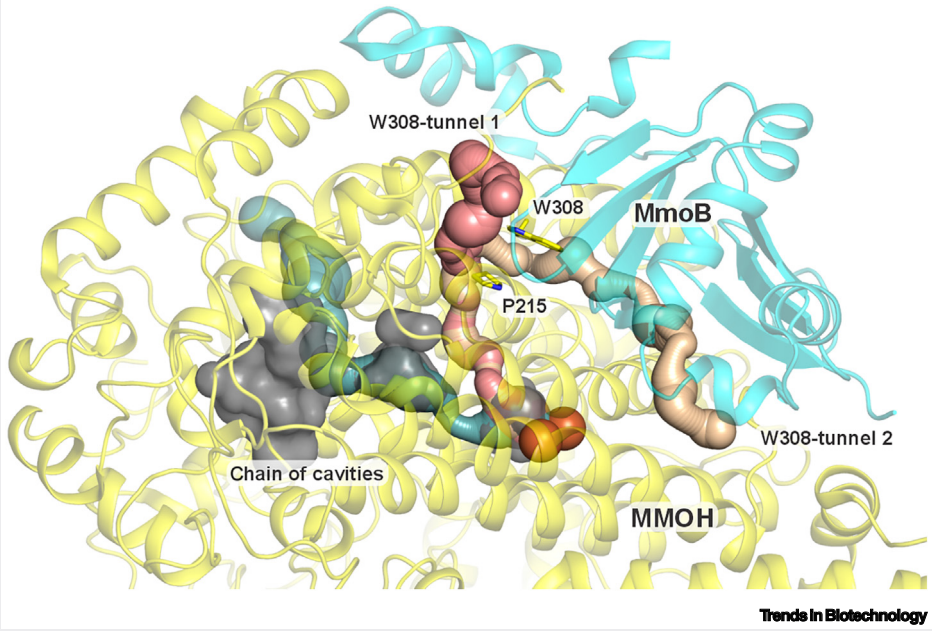

Figure I. Substrate cavities and tunnels of soluble methane monooxygenase (sMMO). The proposals for substrate access routes into the active site cavity are shown in a crystal structure of the reduced MMOH protein in complex with MmoB [Protein Data Bank: 6YDI]. The two versions of the W308 tunnels are named after the gating P215 and W308 residues at the MMOH:MmoB interface. The chain of cavities access route (cyan spheres) threads its way through large cavities (gray surface) within MMOH [111].

Table 2. sMMO derivatives

sMMO host organism	Mutation	Performance	Refs
Methylococcus capsulatus (Bath) sMMO	MmoX^{H246A}	Disruption of [FeFe] coordination inactivates sMMO activity	[112]
	MmoX^{V23G}, MmoX^{T356G}, MmoZ^{R70E}, MmoB^{Y139S}	Presumed enhancement of solubility and expression of sMMO in heterologous host *Escherichia coli*	[112]
	MmoC$^{E56Q/E91Q}$	Reduced sMMO activity while retaining NADH oxidation activity of MmoC. Double mutant still forms cross-links to MMOH, indicating that other cross-linking sites exist, yet disrupted site remains relevant for sMMO complex formation	[113]
Methylosinus sporium 5 sMMO	Δ*mmoX*1, Δ*mmoX*2	Deletion of *mmoX*1 in sMMO operon diminished sMMO activity; deletion of single *mmoX*2 did not impair sMMO activity	[114]
Methylosinus trichosporium OB3b sMMO	MmoX$^{C151E/C151Y}$	Could not be produced at high levels	[115]
	MmoX^{T213S}	Diminished propylene oxidation activity	[115]
	MmoX^{L110G}, MmoX^{L110C}, MmoX^{L110R}, MmoX^{L110Y}	Changed regioselectivity	[37]
	MmoX^{F192}	Changed regioselectivity, which resides close to [FeFe] center and to R98, which is part of hydrogen bonding network proposed to modulate access to cavity pathway	[36]
	MmoB$^{N107G/S109A/S110A/T111A}$ (Quad)	Relaxed size-selective entry of hydrocarbon substrates	[116]
	MmoB$^{N107G/S110A}$ (DBL1)	Caused a 150-fold decrease in formation rate constant of reaction cycle intermediate P	[36]
	MmoB$^{S109A/T111A}$ (DBL2)	Accelerated reaction of [Fe^{IV}Fe^{IV}] intermediate Q with substrates larger than CH_4 by three- to fourfold	[36]
	MmoB^{H33A}	Greatly slowed formation of reaction cycle intermediate P	[36]
	MmoB^{H5A}	Modestly slowed both formation of [Fe^{IV}Fe^{IV}] intermediate Q and its reactions with substrates	[36]
	MmoB$^{\Delta 2-29/}$ V41R	Disruption of N-terminal region retarded O_2 binding to active site	[110,117,118]

Natural methylotrophs have evolved efficient mechanisms to utilize methanol as their sole carbon and energy source. However, limited understanding of their metabolic networks and the scarcity of genetic tools have hindered their development to further expand their use as versatile industrial platforms [74]. To circumvent these challenges, recent strategies focus on implementing synthetic methylotrophy in model organisms, such as *E. coli*, which provide well-established genetic toolkits and proven industrial compatibility (Box 2). However, the establishment of methylotrophy faces significant challenges, such as methanol toxicity at high methanol concentrations and unfavorable thermodynamics [75]. Despite these limitations, *E. coli* serves as a promising chassis for synthetic methylotrophy, involving the heterologous production of methanol oxidoreductases and formaldehyde assimilation pathways.

Box 2. Growth-coupled engineering as a tool for synthetic C1 assimilation

A powerful approach to facilitate the development of synthetic methylotrophy is **growth-coupled engineering**, wherein cell growth is linked to the introduced methanol assimilation pathway. Under this regime, growth serves as a proxy for both enzyme production and activity, enabling indirect selection of improved pathway performance. Modularizing the pathway into functional units allows each module to be screened for activity using growth as a readout, facilitating iterative optimization and identification of rate-limiting steps [119]. These growth-coupled biosensor strains also provide scalable *in vivo* platforms for enzyme screening, because cell fitness depends on enzyme performance, eliminating the need for high-throughput *in vitro* screening [120]. Thus, growth coupling is a powerful tool for developing synthetic methylotrophy and simplifying screening of methanol oxidoreductases and functional modules of C1 assimilation pathways. Combining this approach with adaptive laboratory evolution enables the required global rewiring of the metabolic network [119,120].

Several studies have demonstrated the feasibility of growth-coupled synthetic methylotrophy by engineering strains in which methanol oxidation and formaldehyde assimilation are essential to produce an essential metabolite, or for complete biomass formation. In one case, a formaldehyde-dependent strain was shown to have sufficiently active methanol dehydrogenase to complement methionine **auxotrophy** [121]. In another, the complete biomass was derived from methanol, creating a fully synthetic methylotroph. Several success reports of fully synthetic methylotrophs in model organisms, such as *E. coli* and *S. cerevisiae*, have been published [70,77,78,94]. While these studies clearly demonstrate the feasibility of engineering synthetic methylotrophy, the path to reliably achieve robust and industrially relevant methylotrophic growth remains complex and not yet fully defined. Overcoming current limitations requires a combination of strategies. These include increasing gene copy numbers of key enzymes, using *in vivo* hypermutation systems, applying **rational protein engineering**, and utilizing **directed evolution** methods, such as DNA shuffling and error-prone PCR, to optimize methanol oxidoreductase activity [77,78,94,122].

Ultimately, synthetic methanol-dependent sensor strains can serve as an ideal starting platform for expanding substrate use toward CH_4. Strains that already efficiently convert methanol into biomass are ideal chassis for introducing sMMO. In this context, the methylotrophic chassis becomes a biosensor for CH_4 oxidation. Thus, these strains offer a powerful and scalable platform for functional evaluation and directed evolution of sMMO, accelerating progress toward synthetic methanotrophy and expanding the toolkit for C1 based bioproduction.

The first step, oxidation of methanol to formaldehyde, can be catalyzed by three different enzymes, NAD^+-dependent methanol dehydrogenase (NAD^+-dependent MDH), pyrroloquinoline quinone-dependent methanol dehydrogenase (PQQ-dependent MDH), and O_2-dependent methanol oxidase (MOX) [76–78]. The cofactor has a crucial role in the redox potential of the oxidation step and, thus, its application. While using O_2 as electron acceptor is thermodynamically favorable ($\Delta G'^m = -98.9$ kJ mol^{-1}, assuming a standard concentration of 1 mM for substrates and products), an electron pair is lost because it cannot be used to generate reducing equivalents, more precisely NAD(P)H or to subsequently produce ATP. By contrast, PQQ and NAD^+ are thermodynamically less favorable electron acceptors ($\Delta G'^m = -32.6$ kJ mol^{-1} and $\Delta G'^m = 33.6$ kJ mol^{-1}, respectively). Heterologous production of PQQ-MDH has not yet been accomplished, due to the complex nature of the operon, involving several periplasmatic proteins. Thermodynamically, PQQ-dependent oxidation remains highly favorable under physiological standard conditions, in contrast to NAD^+-dependent oxidation, which is not only thermodynamically the least favorable, but also favors the reduction direction. Nevertheless, NAD^+-dependent MDH is the main focus of research, due to practicability, circumventing thermodynamic constraints by supplying high methanol concentrations [79]. For a detailed review of methanol oxidoreductase, see [79,80]. The second step is the assimilation of formaldehyde, or the more reduced C1 compounds formate, CO, or CO_2, into biomass using several different natural or synthetic metabolic pathways.

Synthetic C1 assimilation routes

Methylotrophs and methanotrophs naturally utilize three primary **C1 assimilation** pathways: the ribulose monophosphate (RuMP) cycle, the serine cycle, and the Calvin–Benson–Bassham (CBB) cycle. *Gammaproteobacteria* predominantly use the RuMP pathway, *Alphaproteobacteria* rely on the serine pathway, and *Verrucomicrobia* fix CH_4-derived CO_2 via the CBB cycle [81–85]. Eukaryotic methylotrophs have an additional methanol assimilation pathway, the xylulose

monophosphate (XuMP) pathway [86]. A shared metabolic feature of the XuMP, RuMP, and CBB pathways is the assimilation step, where the C1 compound is bound to a pentose sugar. The subsequent cleavage and rearrangement steps then differ, involving distinct metabolites from **central carbon metabolism** [87]. In contrast to direct assimilation of formaldehyde via the XuMP and RuMP pathways, the serine cycle first oxidizes formaldehyde to formate, which is incorporated into central metabolism [88]. These distinct mechanisms highlight the diversity among C1 assimilation strategies, because each pathway differs in not only its structural architecture, but also its ATP requirements, the biomass precursors they produce, and their **energetic efficiency**.

In recent years, several natural and synthetic C1 assimilation pathways have been successfully expressed in industrial host organisms, such as *Saccharomyces cerevisiae* and *E. coli* [70,77,78]. Synthetic pathway design aims to complement natural pathways by accelerating the emergence of novel assimilation routes. Through heterologous expression, which mimics the natural mechanism of horizontal gene transfer, the design space of possible pathways is expanded. This encompasses the full repertoire of known enzymatic reactions across all domains of life [89].

For synthetic C1 assimilation suitable for synthetic methylotrophs and methanotrophs, formaldehyde conversion to biomass has a crucial role in providing carbon to central metabolism. Rapid formaldehyde assimilation is essential due to its high toxicity [70,90]. One synthetic approach for formaldehyde assimilation is the synthetic acetyl-CoA pathway (SACA), which condenses two formaldehyde molecules into acetyl-CoA [91]. Although this synthetic formaldehyde assimilation pathway offers high theoretical **carbon yields** *in vitro*, its *in vivo* implementation is limited, with only 3% of biomass coming from methanol, which was fed as a co-substrate together with yeast extract and tryptone. This is largely due to suboptimal enzyme kinetics and affinities of key components, such as glycolaldehyde synthase [91,92]. A subset of synthetic methylotrophic pathways is shown in Figure 3 (Key figure), all of which were reported to contribute to methanol-derived biomass.

Another example of a synthetic pathway predicted to outperform its natural counterpart is the homoserine cycle. Flux–balance analysis predicted a 13% increase in biomass compared with the serine cycle. The homoserine cycle has two formaldehyde entry points: one leading to serine and the other to hydroxy-2-oxo-butanoate. While partial incorporation of methanol-derived formaldehyde was demonstrated, fully establishing the cycle was not achieved [93]. A more recent strategy similar to the SACA pathway is the synthetic methylotrophic assimilation (SMA) pathway. Two formaldehyde molecules are first condensed to form glycolaldehyde, which is subsequently incorporated into hexose-6-phosphate. This pathway has been successfully demonstrated in *E. coli*, where complete biomass formation from methanol was achieved, effectively establishing a fully synthetic methylotrophic strain [94]. Another notable example is the synthetic reductive glycine pathway (rGlyP), initially designed as a highly efficient route for formate assimilation. This pathway was later extended to enable methanol assimilation, serving as both carbon and energy source [71]. While the strains with rGlyP exhibit a doubling time of 54 h, the SMA pathway was shown to enable a considerately faster doubling time of 4.5 h, operating at a growth rate close to that of natural methylotrophs. A recent review quantitatively assessed different natural and synthetic C1 assimilation pathways, evaluating their maximal theoretical yields for the bioproduction of various compounds [95]. For some of the studied products, the synthetic C1 assimilation pathways surpass natural ones in terms of product yield. Notably, the yield increases with the degree of reduction, showcasing CH_4 as a promising substrate for bioproduction. This highlights the potential of synthetic C1 assimilation pathways as viable alternatives to natural routes, offering expanded metabolic flexibility and tunability for applications such as bioproduction.

Key figure

Overview of synthetic one-carbon (C1) assimilation pathways

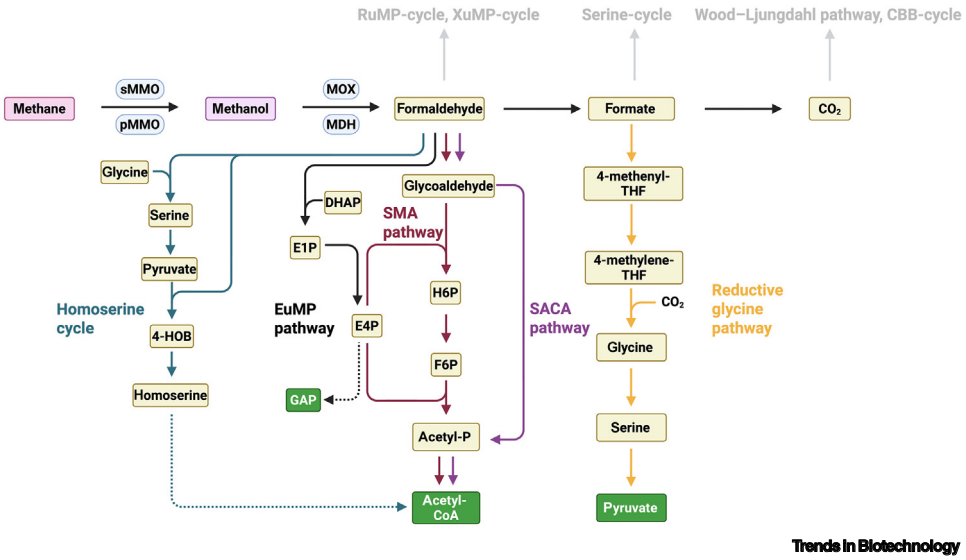

Figure 3. The schematic depicts multiple theoretical synthetic C1 assimilation pathways that could be implemented to establish synthetic methanotrophy in non-native hosts. Methane is oxidized by soluble or particulate methane monooxygenases (sMMO/pMMO) and methanol by methanol oxidoreductases (MOX/MDH), both together generating formaldehyde as a central starting molecule for various C1 assimilation pathways. Depending on the engineered C1 pathway [homoserine cycle, erythrulose monophosphate pathway (EuMP) pathway, synthetic methylotrophic assimilation (SMA) pathway, synthetic acetyl-CoA (SACA) pathway, or reductive glycine pathway], methane (CH_4) is converted through distinct routes to metabolites that serve as entry points into the central metabolism of the non-native host. Colored arrows indicate enzymatic steps within the respective synthetic C1 pathways, while gray arrows depict natural C1 assimilation routes. Green-colored metabolites mark the entry points into central carbon metabolism of the non-native host. Broken arrows represent sequences of multiple enzymatic reactions. Abbreviations: 4-HOB, 4-hydroxy-2-oxobutanoate; 4-methenyl-THF, methenyl-tetrahydrofolate; 4-methylene-THF, methylene-tetrahydrofolate; acetyl-CoA, acetyl coenzyme A; acetyl-P, acetyl phosphate; CBB, Calvin–Benson–Bassham cycle; DHAP, dihydroxyacetone phosphate; E1P, erythrose-1-phosphate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; GAP, glyceraldehyde-3-phosphate; H6P, hexulose-6-phosphate; MDH, methanol dehydrogenase; MOX, methanol oxidase; RuMP, ribulose monophosphate pathway; XuMP, xylulose monophosphate pathway.

Outstanding questions

What is the physiological role of the chaperonin MmoG and maturase MmoD with respect to sMMO maturation in non-native hosts?

How can we protect overoxidation of methanol by sMMO and toxicity effects?

How can we further overcome solubility issues in heterologous production of sMMO in non-native hosts?

How does sMMO integrate into the native metabolism of non-native hosts in terms of redox balance and how can this interplay be optimized?

How can we apply growth-coupled protein engineering of sMMO in methylotrophic biosensor strains to identify beneficial mutant variants?

Can alternative CH_4-oxidizing enzymes be further engineered toward the design of synthetic methanotrophy?

Can formaldehyde toxicity be addressed by producing methanol oxidoreductase in bacterial microcompartments or eukaryotic organelles?

How can we design CH_4-based bioprocesses to prevent limitation of gaseous substrates and simultaneously facilitate online monitoring of dissolved CH_4 and safe gas atmospheres?

Concluding remarks and future perspectives

The development of robust synthetic methanotrophs holds significant promise for sustainable biotechnological applications. With the increasing urgency to utilize CH_4 as a renewable resource, modern strain engineering efforts are now focused on transferring CH_4-assimilating pathways into genetically tractable and industrial host organisms, such as *E. coli*. This synthetic methanotrophy approach provides a more versatile and genetically accessible platform for the integration of complex biosynthetic pathways, circumventing the challenges associated with the limitations posed by the metabolic rigidity and genetic intractability of native methanotrophs, thereby facilitating more efficient development of CH_4-based bioconversion processes. However, integrating synthetic metabolic pathways into the central metabolism of microbial hosts remains challenging. Cofactor balance, O_2 tolerance, complex folding and maturation of biocatalysts, and metabolic burden are persistent limitations that must be addressed.

Recent advances in the discovery and engineering of CH_4-oxidizing biocatalysts, including canonical sMMOs, CYP monooxygenases, MCRs, and synthetic *de novo* monooxygenases, such as mini-sMMO, represent the growing power of protein engineering to tailor CH_4-oxidizing enzymes for function in heterologous systems. Significant progress has been made in heterologous sMMO production in *E. coli*, supported by the use of chaperones, protein engineering, and solubility-enhancing derivatives. Looking ahead, future research should prioritize high-throughput protein-engineering strategies, with particular emphasis on generating diverse sMMO mutant libraries. Improving protein yield in the soluble proteome fraction of *E. coli* and developing high-throughput screening assays will be essential to identify sMMO variants that confer tractable CH_4-oxidzing activity in non-native hosts.

Parallel progress in implementing engineered formatotrophy and methylotrophy in *E. coli* underscores the feasibility of redirecting carbon flux through non-native C1 pathways, providing a foundation for the future development of methanotrophic chassis. Ongoing efforts in engineered methylotrophy in industrial hosts have enhanced microbial capacity to sustain C1 metabolism, with notable successes including faster growth rates solely on methanol. Building on this foundation, methylotrophic sensor strains are expected to serve as valuable tools for engineering of optimized sMMO variants, thereby advancing synthetic methanotrophy. In this context, the quantitative investigation of synthetic methylotrophic metabolism in *E. coli* by metabolic flux analysis (MFA) may provide key insights into how C1 pathways are integrated into the central metabolism of non-native hosts. Such understanding will be essential for identifying metabolic targets to redirect and optimize carbon flux. However, isotopic labeling with gaseous C1 substrates, namely CH_4, remains technically challenging and resulting MFA patterns are complex to interpret. The aim of realizing efficient CH_4 assimilation in industrial hosts will likely require adaptive laboratory evolution to improve growth and pathway robustness. Moreover, setting realistic performance benchmarks is critical: while, in batch cultures, native methanotrophs reach average maximum CH_4 uptake rates of ~18.6 mmol g CDW^{-1} h^{-1}, even achieving ~20% of this capacity in a non-native host would represent a major step toward practical implementation of synthetic methanotrophy [96,97].

In addition, realizing the full potential of synthetic methanotrophy also demands advances in gas-based bioreactor design to consolidate increased substrate availability (e.g., via pressure), safety concerns for explosive gas mixtures, and online monitoring of gas transfer rates and dissolved gas concentrations. These factors make gas fermentation overall more challenging and represent a critical aspect to establishing utilization of C1 compounds, such as CH_4, as substrates. Overcoming these challenges is essential to enable the transition from laboratory and pilot-scale setups to large-scale industrial bioreactors, thereby facilitating the commercial application of CH_4-based biotechnologies.

The synergy of synthetic biology, enzyme engineering, and bioprocess technology offers a promising framework to convert CH_4 into valuable chemicals. Nevertheless, several key challenges remain (see Outstanding questions) and will require interdisciplinary solutions to translate these concepts into scalable industrial processes.

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Declaration of interests

The authors declare no competing interests.

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