Methane Biocatalysis: Selecting the Right Microbe

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Abbreviations

AMO	arobic methane oxidation
ANME	anaerobic methanotrophic archaea
AnMO	anaerobic methane oxidation
BS	bifidobacterium shunt
CBB	Calvin-Benson-Bassham cycle
CCE	carbon conversion efficiency
DCW	dry cell weight
EDD pathway	Entner-Doudoroff pathway
EMP pathway	Embden-Meyerhof-Parnas pathway
MCC	methanol condensation cycle
PHB	polyhydroxybutyrate
RuMP pathway	ribulose monophosphate pathway
SC	serine cycle
pSC	partial serine cycle
SCP	single-cell protein
TCA	tricarboxylic acid cycle
rTCA	reverse tricarboxylic acid cycle
WLP	Wood-Ljungdahl pathway

13.1 Introduction

Methane-oxidizing bacteria (methanotrophs) are natural systems for the attenuation of methane emission. These microbes can use methane as their sole source of energy and carbon and thus are unique systems for methane-based bioconversions. The industrial potential of methane metabolism has actively been discussed for almost a half a century. Methanotrophic cultures have been tested for the production of single-cell protein (SCP),[1,2] poly-β-hydroxybutyrate (PHB),[3] components for nanotechnological applications,[4] lipids,[5] and carotenoids[6] and as a carbohydrate feedstock for fermentation.[7,8] A number of methane-driven transformations, such as epoxidation, calcium carbonate precipitation, bioleaching, biofiltration, and bioremediation, have also been explored.[9–15] However, only the production of methanotrophic SCP (such as BioProtein/Norferm, Norway,

http://calysta.com; UniProtein/Unibio, Denmark http://www.unibio.dk, and Gaprin, Russia, http://atelgroup.org/en/biotechnology) using a mixed culture with *Methylococcus capsulatus* has remained a commercially established product. A number of factors contributed to the slow progress in methane-based biotechnology, including the increased energy prices in the late 1970s to early 1980s, which reduced the attractiveness of natural gas as a carbon feedstock. By the early 1990s it had already been concluded that the future of C_1 biocatalysis lays in the production of high-cost products,[16] although difficulties in the genetic reprogramming of methanotrophic bacteria together with fairly cheap chemical fossil-based synthesis almost completely eliminated the attention given to bioconversions.

Resurgence of interest in the industrial application of methane biocatalysis is occurring now. The energy market landscape (cheap natural gas *vs* expensive oil) and energy boom in the United States are most likely the main drivers of the new wave of attention being given to methane.[4,5,17–19] However, history shows that this interest can easily decline in parallel with oil prices. Experts in the field believe that new methane-based technology must go beyond short-term economic considerations and focus on long-term sustainability. It is important to remember that methane is a very controversial source of energy—one which comes with an exceptionally large environmental footprint.[20,21] Anthropogenic methane emission accounts for more than 60% of the greenhouse gas budget and it is the number one target for global climate regulation.[22] Fossil fuel production (e.g., underground coal mining, oil-associated gas, and natural gas pipelines/compressors) is the first target for new technologies. However, the technology can also benefit other areas, such as agriculture (e.g., enteric fermentation in livestock, manure management, and rice cultivation), landfills, municipal wastewater, and rising biofuel production.[23,24] Up to 116 million tons of oil-associated methane and 40 million tons of biogas (equivalent to 27% of the total United States transportation fuel) are flared each year. Any gas flare, from small to large, represents lost energy, lost revenues, and unnecessary CO_2 emissions. Gas flares are also a dangerous source of air pollution. A search for new environment-friendly approaches for methane capture is an ongoing journey, and it is not yet apparent which innovation might represent the best solution. If developed, methane biocatalysis could fill this niche and the technology would certainly withstand future oil-price drops and rises.

Microbial biotechnology is grounded on the fundamental knowledge of related systems. Our understanding of methane utilization has changed dramatically in recent years. A number of novel microbial strains with novel metabolic arrangements of methane utilization have been described. The new microbes inspire conceptually novel ideas for the intensification of methane conversion and stimulate new elaborations in the area. This chapter summarizes the most recent development in microbial methane biocatalysis. Several strategies are outlined below for streamlining the selection of microbial systems for biotechnological alterations. These include metabolic considerations and the physiological advantages or constraints of specific microbial cultures.

13.2 Methane Biocatalysis: Basic Concepts

Two fundamentally different mechanisms of methane utilization are known: oxygen dependent (mostly aerobic) and oxygen independent (anaerobic). Aerobic methane oxidation (AMO) relies on oxygen for methane activation and oxidation and has thus far been described for Proteobacteria, Verrucomicrobia, and NC10 phyla. Oxygen-independent methane oxidation is based on reverse methanogenesis. A number of anaerobic methane oxidation (AnMO) couplings have been proposed.[25,26]

So far only aerobic fermentation with methane has been proven at the large scale, demonstrating that safe and efficient operation is achievable. However, when realized at the industrial scale, methane catalysis appeared to be more expensive then was initially projected.[16] One of the overlooked economic aspects of methane-based conversion could have been the cost of oxygen, the second compound required for the process. Today a number of alternate strategies for methane conversion, which engage nitrate, sulfate, or three metal oxides to fulfill oxygen's functions, are being discussed. Below, the cost of each conversion is estimated based on the current prices of the nutrients required for methane oxidation.

Aerobic methane conversion requires an oxygen input with at least a 1:1 methane to oxygen ratio. Oxygen is widely available, but it is a pricy feedstock. The cost of oxygen is directly linked to energy cost (mostly electricity) and might vary from $100 to $125 per ton.[27] That estimation would bring the cost of aerobic methane conversion to at least $200-$250 per one ton of methane (Figure 13.1). In fact, high cell growth would actually require higher oxygen input (at 1:1.5-2 methane to oxygen ratio) making the cost of AMO conversion even higher, up to $375-$500 per ton of methane.

The cheapest AnMO process is the one linked to sulfate reduction (SR). With the price of sulfuric acid ranging from $25 to 100 per ton (www.icis.com), the AnMO-SR conversion would cost $30-$600 per ton of methane; the price would be much higher ($1000-$1500) if sulfates are used. AnMO supported by denitrification (which requires 4 mol of nitrate per 1 mol of methane oxidized) is about $3400 per ton of methane if nitric acid is used [at the price of $215-$225 per ton (www.icis.com)] and at least $7600 per ton if sodium nitrate is used. AnMO supported by metal oxides comes with a remarkably high price tag (Figure 13.1).

Other factors challenging the applicability of AnMO-based technology which are rarely discussed in the literature are its overall process sustainability and possible environmental impact. The current markets for sulfuric acid/sulfates, nitric acid/nitrates, and metal oxides are quite narrow, and they would have to be expanded significantly to support methane catalysis. For example, the conversion of flared oil-associated methane (estimated to be at least 116 million tons) would require 696 million tons of sulfate, but the global annual production of sulfuric acid is only 200 million tons (http://www.essentialchemicalindustry.org) while sodium sulfate is only 16-17 million tons (http://www.cefic.org).
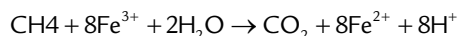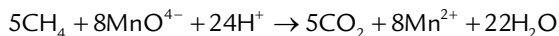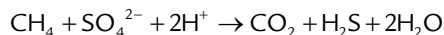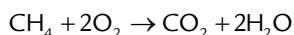
Figure 13.1

Overview of aerobic and anaerobic methane oxidation: microbes, parameters, by-products, and economics. DCW, dry cell weight; MO, methane oxidation; RuMP, assimilatory ribulose monophosphate pathway; SC, serine cycle; EMP, ethylmalonyl-CoA pathway; CBB, Calvin cycle; WLP, the reductive acetyl-CoA (Wood-Ljungdahl) pathway; *, pathway is present only in some species. Cost of methane conversion was estimated based on the methane conversion equations (25-26, and below) and current price of electron acceptors (taking from www.icis.com).

$$CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$$

$$CH_4 + SO_4^{2-} + 2H^+ \rightarrow CO_2 + H_2S + 2H_2O$$

$$CH_4 + 4NO_3^{3-} \rightarrow CO_2 + 4NO^{2-} + 2H_2O$$

$$5CH_4 + 8MnO^{4-} + 24H^+ \rightarrow 5CO_2 + 8Mn^{2+} + 22H_2O$$

$$CH4 + 8Fe^{3+} + 2H_2O \rightarrow CO_2 + 8Fe^{2+} + 8H^+$$

Another aspect that challenges the AnMO process is that intensive oxidation of methane requires significant input of sulfate or nitrate into the cultivation environment. Assuming that the rate of AnMO is in the same range at AMO, or about $20\,mmol\,h^{-1}\,g^{-1}$ dry cell weight (DCW), a bioreactor with about 30 g DCW per liter would consume sodium sulfate at the rate $58.8\,g\,h^{-1}\,L^{-1}$ and sodium nitrate at the rate $37.2\,g\,h^{-1}\,L^{-1}$. That indicates that the AnMO-based

process has to be built using strains capable of tolerating high salt (halophiles) or, if electron acceptors are added as acids, extremely low pH (acidophiles). That would remain true even for an alternate host of the AnMO modules.

Even with a very cursory look, AnMO-based technology might have dramatic environmental impacts. The ANMO-SR mode is directly linked with the formation of volatile sulfur compounds, such as H_2S. This mode of methane oxidation would not be feasible for technological applications unless a sustainable solution for conversion of H_2S back to sulfate or elemental sulfur is found. In theory, that could also be accomplished via biological pathways, such as photosynthetic sulfur oxidation linked to light, oxygen, or nitrate.[28] The AnMO-DR system might leak toxic intermediates and dangerous greenhouse gases, such as NO, N_2O, and ammonia. Like SR, some impacts of the denitrification process could be complimented by additional (bio)technological modules, such as coupling to ammonium oxidation.[29] That, however, also requires additional breakthrough developments in areas beyond methane biocatalysis.

Taking into account all three parameters discussed for AnMO-based technologies (cost, sustainability, and environmental impact), AMO remains the more feasible solution for a methane-based technology if it is considered for a wide deployment. However, the AnMO process might be beneficial for some areas. For example, methane-activated denitrification might find its way to the market as an innovative solution to wastewater treatment, and perhaps some biomass produced in the process could be converted into value-added chemicals. In the long term, feasibility of AnMO will depend on breakthrough developments in synthetic biology. While it is hard to predict what innovation might bring advanced methane biocatalysts, one aspect of the AnMO-based technology is apparent—it has to be accomplished only as a closed, self-sustained cycle.

13.3 Selecting the Right Microbe: C_1-Oxidation Options
13.3.1 Methane Oxidation—Aerobic

Methane oxidation is driven by mixed function oxidases, in which one atom from O_2 goes to methanol and the other to water, requiring the input of two electrons and two protons[30]; Figure 13.2a).

Two isoforms of the enzyme are described: cytosolic soluble methane monooxygenase (sMMO) and membrane bound, or particulate methane monooxygenase (pMMO).[31] sMMO uses NADH as its electron donor[32]; the physiological electron donor to pMMO is still not known, but it is widely accepted that reducing equivalents produced by formaldehyde and/or formate oxidation (NADH or QH_2) are required for its enzyme activity.[30] In all known methanotrophs, the second step of the AMO pathway is catalyzed by a periplasmic pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase (MDH). Two systems are described: a two subunit enzyme, known as Mxa, encoded by *mxaFI* genes[33,34] and a
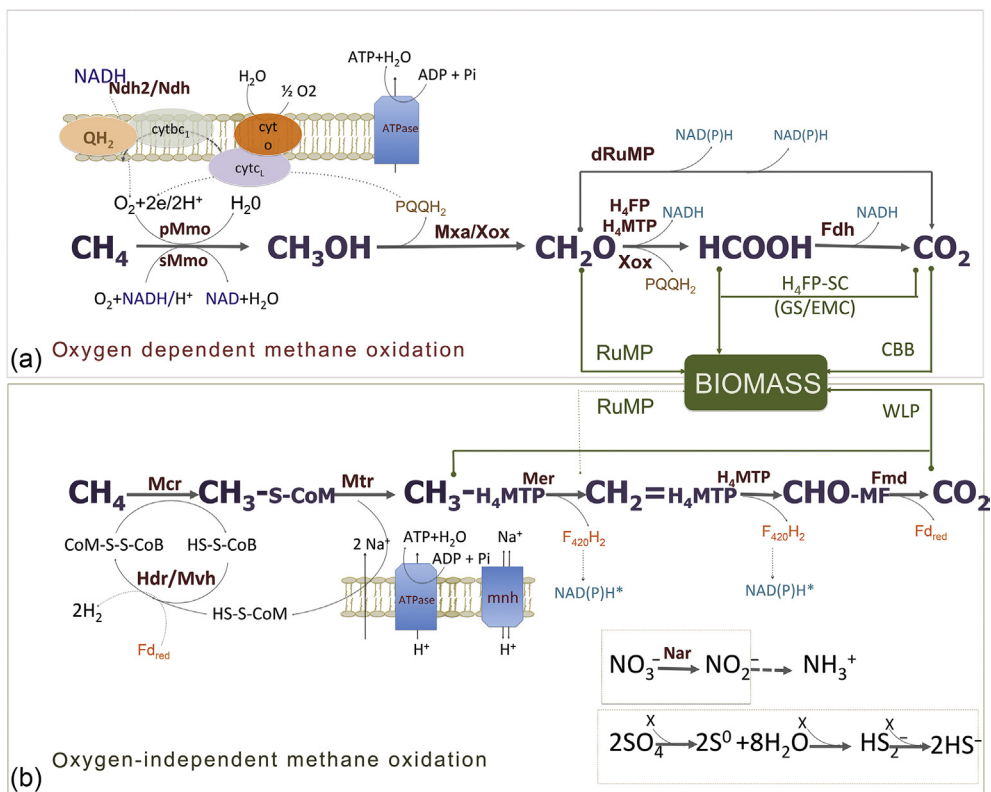
Figure 13.2

Schematic illustration of methane metabolism. (a) Aerobic methane utilization; (b) anaerobic methane utilization. pMMO, membrane-bound methane monooxygenase; sMMO, soluble methane monooxygenase; Mxa, PQQ-linked methanol dehydrogenases; Xox, PQQ-linked methanol and formaldehyde dehydrogenases; H_4MTP, methanopterin-linked C_1 transfer; H_4FP, folate-linked C_1 transfer; Fdh, formate dehydrogenases; NDH2, NADH:quinone oxidoreductase; dPPP, dissimilatory pentose phosphate cycle; RuMP, assimilatory ribulose monophosphate pathway; SC, serine cycle; EMP, ethylmalonyl-CoA pathway; CBB, Calvin-Benson-Bassham cycle.

monosubunit enzyme, encoded by *xoxF* genes.[35] Both systems are associated with cytochrome *c* L (*mxaG* or *xoxG*) and a protein of unknown function protein (*mxaJ* or *xoxJ*), which enhances the activity of the enzyme.[36,37] The methanol oxidation machinery also includes a number of proteins for the enzyme assembly and biosynthesis of PQQ.[34,38]

While several formaldehyde dehydrogenases were reported in early studies of methanotrophs,[39–42] so far, only the presence of the broad-specificity housekeeping aldehyde dehydrogenases has been confirmed by genomics.[43–46] The aldehyde dehydrogenases show quite low expression levels upon growth on methane and their role in primary C_1 oxidation remains to be validated.[47,48] However, it has been shown that the Xox-type MDH also contributes to oxidation of formaldehyde in Verrucomicrobial methanotrophs.[49] As *xox* genes

are quite widespread among bacteria,[49,50] it is possible that the Xox enzyme plays at least some role in formaldehyde oxidation in all methanotrophs. Among the pathways commonly predicted for formaldehyde conversion in methanotrophic bacteria are the oxidation through H_4folate or H_4methanopterin intermediates[1,51] and the dissimilatory ribulose monophosphate phosphate (dRuMP) pathways.[52] The cofactor-linked pathways generate 1 mol of NADH per 1 mol of formaldehyde converted to formate. The dRuMP pathway leads to the production of 2 mol of NAD(P)H and 1 mol of CO_2 per 1 mol of formaldehyde. Formate oxidation is typically carried out by NAD-dependent formate dehydrogenases containing molybdenum or tungsten.[45,48,53]

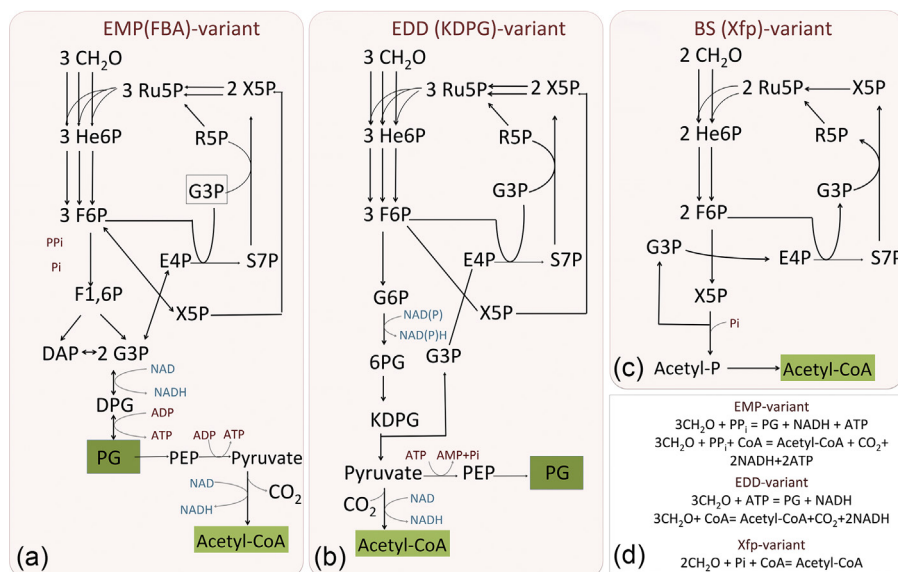### 13.3.2 Methane Oxidation—Anaerobic

The energetic and environmental parameters of ANMO have recently been reviewed by Caldwell et al.[25] ANMO coupled with the reduction of nitrate,[54] sulfate or elemental sulfur,[26,55–57] or metals[58] are discussed in this chapter, as they are the only naturally occurring reactions that have been at least partially defined on a biological level. AnMO is typically driven by a mix culture (or consortia) of anaerobic methanotrophic archaea (ANME) and bacteria, rather than a pure culture. Because none of the anaerobic methanotrophs have been isolated in pure culture or coculture, not much is known about pathways and enzymes involved in primary methane oxidation and assimilation. The key kinetic parameters of the most typical arrangements of AnMO are summarized in Figure 13.1. The proposed reverse methanogenesis is supported by remarkable enzymatic studies on methyl-CoA reductase (MCR), which also carries out biological methane formation.[59] The arrangement of metabolic reactions downstream from the MCR step remains elusive. It is assumed that methane-oxidation products follow the reverse methanogenic pathways all the way back to CO_2 or acetate (Figure 13.2b).[54,60]

13.3.3 Methane Oxidation—Summary

A summary of the core enzymes involved in methane oxidation is shown in Figure 13.2. Based on available data, complete AMO can be achieved by only three enzymes: MMO, Xox-type methanol/formaldehyde dehydrogenase, and formate dehydrogenase. This most simplistic configuration of methane utilization would require three genes for pMMO, at least three genes for methanol and formaldehyde oxidation—assuming that the host has the PQQ biosynthesis inventory (nine genes if it does not)—and at least two genes to complete the conversion of formate into CO_2 and to generate reducing power.

The rates and efficiencies of methane oxidation might vary quite significantly even among closely related species; however, some common trends can be noted. For AMO, typically cells expressing pMMO show higher growth rates and higher carbon conversion efficiencies (CCEs) than cells using sMMO.[61–64] Interestingly, the activity of the enzyme

in archaea/sulfate-reducing bacteria consortia aligns with the lowest parameters observed for aerobic methane oxidizers. The rates of AnMO linked to reduction of sulfates are almost three orders of magnitude higher than the rates of AnMO coupled to denitrification or reduction of metal oxides (Figure 13.1).[54–58,65,66] Since the exact energy requirements for pMMO-based methane utilization are not well established, any *in silico* predictions of methane oxidation efficiencies are to some degree arbitrary. All comparisons provided below are based entirely on assimilation, with the assumption that the energetic inputs and outputs of the primary oxidation machinery are identical among all methanotrophs.

13.4 Selecting the Right Microbe: C_1-Assimilation Options

Natural pathways for single carbon assimilation come in a great variety of metabolic arrangements, which fundamentally can be split into three main categories: *CH$_2$O to biomass conversion*, which occurs via the assimilatory ribulose monophosphate (aRuMP) pathway (Figure 13.3)[50,67]; [CH$_2$] *to biomass conversion*, which involves the serine cycle

Figure 13.3

The ribulose monophosphate pathway (RuMP) in methanotrophic bacteria. (a) EMP (FBA) variant; (b) EDD (KDPG) variant; and (c) BS (Xfp) variant. (d) Summary equations for the production of phosphoglycerate or acetyl-CoA from formaldehyde. DAP, dihydroxyacetone phosphate; DPG, 2,3-diphosphoglycerate; E4P, erythrose-4-phosphate; F6P, fructose 6-phosphate; F1,6P, fructose 1,6-bisphosphate; G3P, glyceraldehyde 3-phosphate; G6P, glucose 6-phosphate; He6P, 3-hexulose 6-phosphate; KDPG, 2-keto-3-deoxy 6-phosphogluconate; 6PG, 6-phosphogluconate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; S7P, sedoheptulose 7-phosphate; X5P, xylulose 5-phosphate; PPi, diphosphate; Pi, inorganic phosphate.
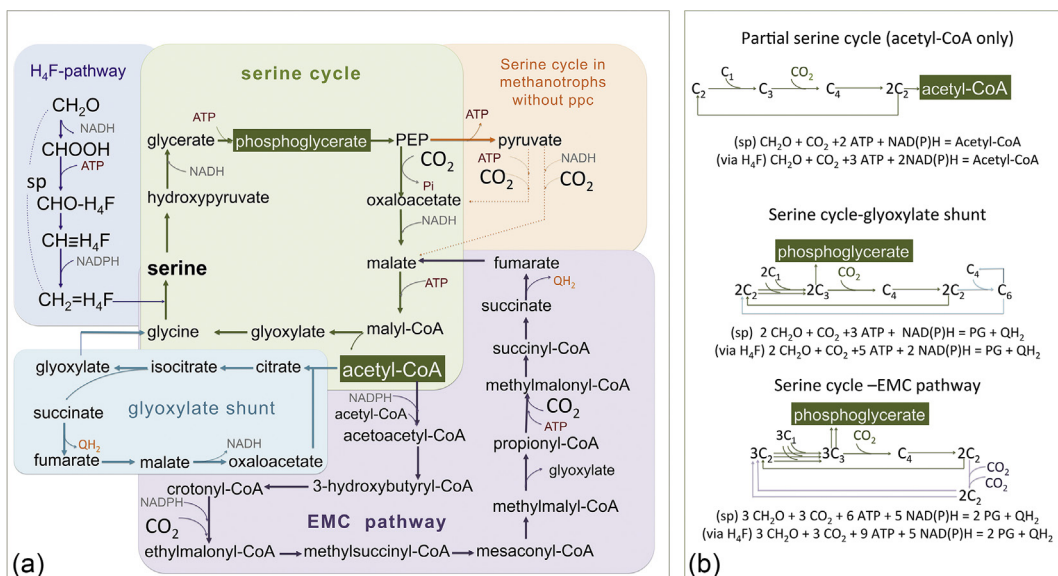
Figure 13.4

Overview of the metabolic arrangement for $[CH_2]$- to biomass conversion. (a) The serine cycle and associated reactions in methanotrophic bacteria. (b) Summary equations for the production of phosphoglycerate or acetyl-CoA from formaldehyde. sp, spontaneous condensation of formaldehyde on H_4-folate.

(SC), at least one of the glyoxylate regeneration pathways, and, under certain circumstances, tetrahydrofolate (H_4F)-linked C_1 transfer (Figure 13.4)[68–71]; and *CO$_2$ to biomass conversion*, which theoretically could cover all the described CO_2-fixation pathways.[72,73] Different microbes show different inventories for assimilation, and some of them might have pathways for carbon fixation at all three levels (see Table 13.1). The details of methylotrophic assimilatory pathways will be described below.

13.4.1 CH$_2$O to Biomass Conversion

The two key enzymes of the CH_2O to biomass conversion, 3-hexulose 6-phosphate synthase (EC 4.1.2.43, Hps) and 3-hexulose 6-phosphote isomerase (EC 5.3.1.27, Hpi), are responsible for condensation of formaldehyde on ribulose 5-phosphate (R5P) to form hexulose 6-phosphate and its conversion into fructose 6-phosphate. Hps and Hpi are core enzymes for C_1 assimilation in methanotrophic Gammaproteobacteria.[117] Theoretically, three variants of the RuMP pathway could be predicted, including the Entner-Doudoroff pathway (EMP) (glycolytic) variant (also known as FBP variant); the Entner-Doudoroff (EDD) pathway (KDPG variant); and the bifidobacterium shunt (BS) variant (also known as phosphoketolase, Xfp variant, also proposed as a biocatalytic methanol condensation cycle (Figure13.3)).[1,118,119]

Table 13.1: General summary for publicly available methanotrophic cultures

Name	Species[*]	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	Reference
		Growth rate (h⁻¹)	Metabolic pathways	pH	T °C	Other		

Here the "Growth parameters" header spans: Growth rate, Metabolic pathways, pH, T °C, Other.

Name	Species[*]	Growth rate (h^{-1})	Metabolic pathways	pH	T °C	Other	Culture collection ID [Genome Accession Number (NCBI)]	Reference
				Group I				
Methylobacter	*M. luteus*	0.12	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	NA (6.8)	5-55 (37)	Produces yellow water-soluble pigment and bacteriocin-like compounds	**89/VKM B-2111** [NZ_ATYJ00000000.1]	74
	M. marinus	0.15	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	NA (7.2)	5-35 (30)	Grows at modest salinity (0.1-5%)	**A45**, Dr. Lidstrom culture collections (University of Washington) [NZ_ARVS00000000.1]	
	M. tundripaludum	0.02	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	5.5-8 (6.8)	5-30 (23)	Does not grow well on methanol, might be capable of nitrogen fixation	**SV96ᵀ** (= DSM 17260ᵀ = ATCC BAA-1195ᵀ) [NZ_AEGW00000000.2]	75,76
	M. whittenburyi	0.1	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	5-8 (6.8)	5-40 (25)		**UCM-B-3033**, Dr. Romanovskaya V.A. culture collection (Institute of Microbiology, Ukraine) [JQNS01000000]	
	Methylobacter sp.BBA5.1	0.06	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	NA (7.2)	5-35 (30)	Grows at modest salinity (0.1-5%)	**BBA5.1**, Dr. Lidstrom culture collections (University of Washington) [JQKS01000000.1]	
Methylocaldum	*M. szegediense*	0.03	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS) CBB, pSC	NA (6.8)	30-61 (55)	Produces melanin-like pigments, and can excrete 4-hydroxyphenyl acetic acid. Might be capable of PHB production	**O-12**, Dr. Trotsenko Yu.A. culture collection (IBPM) [ATXX01000000]	77,78

Methylococcus	M. capsulatus Bath	0.3-0.4	pMMO, sMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS) CBB, pSC	6-8 (7)	30-55 (42)	Well-established catalyst for SCP production	**Bath^T** (= ACM 3302^T = ATCC 3309^T = NCIMB 11132^T) [NC_002977]	46,79
	M. capsulatus Texas	0.1	pMMO, sMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS) CBB, pSC	6-8 (7)	30-50 (37)	Grows well on methanol	**Texas** (=ACM 1292^T = ATCC 19069^T = NCIMB 11853^T) [NZ_AMCE00000000.1]	80,81
Methylogaea	M. oryzae	NA	pMMO, Mxa, RuMP (EMP,EDD,BS) CBB, pSC	5-8 (7)	20-37 (30)	Grows at low salinity. Might be capable of nitrogen fixation and PHB production	**E10^T** (=JCM 16910^T = DSM 23452^T) [PRJDB848]**	82
Methyloglobulus	M. morosus KoM1	0.14	pMMO, RuMP (EMP,EDD,BS)pSC	5-8.5 (6-8)	4-30 (20)	Can fix nitrogen, prefers to grow at low O2	**KoM1^T** (=DSM 22980) [NZ_AYLO01000180]	83,84
Methylohalobius	M. crimeensis 10Ki	0.03-0.05	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP, BS), CBB, pSC	6.5-7.5	15-42 (30)	Grow optimally at 1-1.5 M NaCl	**10Ki** = DSM 16011^T = ATCC BAA-967^T [NZ_ATXB00000000]	[85]P. Dunfield personal communication
Methylomarinum	M. vadi IT-4	0.33	pMMO, Mxa, H_4MTP, H_4F, RuMP	4.5-7.0 (6.4)	20-44 (37)	Grows well at low pH and relatively high salinity. The strain was isolated from thermal vent and might be tolerant to short-chain alkanes	**IT-4^T** (=JCM 13665^T = DSM 18976^T) [NZ_JPON00000000]	86
Methylomarinovum	M. caldicuralii	0.29-0.33	pMMO, RuMP	5-7 (6-6.4)	30-55 (45-50)	Growth at salinity 1-5% (optimum 3%) and high temperature	**IT-9^T** (=JCM 13666(T) = DSM 19749(T)	87
Methylomicrobium	M. agile A30	0.06	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	5-8 (6.8)	5-30 (25)		**A30** (=ACM 3308^T = ATCC 35068^T = NCIMB 11124^T) [NZ_JPOJ00000000.1]	79
	M. album BG8	0.11	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	5-8 (6.8)	5-30 (25)		**BG8^T** (= ACM 3314^T = NCIMB 11123^T) [NZ_AFJF00000000.2]	88-90

Table 13.1: General summary for publicly available methanotrophic cultures—cont'd

Name	Species*	Growth rate (h^{-1})	Metabolic pathways	pH	T °C	Other	Culture collection ID [Genome Accession Number (NCBI)]	Reference
Methylomonas	M. alcaliphilum 20Z	0.12	pMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	6-9 (8.5)	5-42 (30-37)	Grows well at 1 M NaCl. Resistant to desiccation	FO082060 **20Z**^{T}: VKM B-2133;5G Dr. Trotsenko Yu.A. culture collection (IBPM) [FO082060]	39,45,91,92
	M. buryatense 5G	0.23	pMMO, sMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	6-9 (8.5)	5-45 (30)	Robust growth	**5G** Dr. Trotsenko Yu.A. culture collection (IBPM) [NZ_AOTL00000000.1]	93,94
	M. methanica MC09	NA	pMMO, sMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	NA	NA	Strain was isolated from seawater, and can grow at 1 M salinity	Dr. Murell lab culture collection (University of East Anglia) [CP002738; NC_015572.1]	95
	Methylomonas LW13	0.16	pMMO, sMMO, Mxa, H_4MTP, H_4F, RuMP (EMP,EDD,BS)pSC	NA (6.8)	NA (30)	Strain shows good growth parameters	**LW13**, Dr. Lidstrom culture collections (University of Washington) [PRJNA225845]**	96
	M. paludis	NA	pMMO	3.8-7.3 (5.8-6.4)	8-30 (20-25)	Acid-tolerant methanotroph	**MG30**^{T} = DSM24973^T = VKM B-2745^T	97
	M. lenta	NA	pMMO	6.3-7.8 (6.8-7.3)	15-28 (20)	Strain was isolated from a slurry pit of a cow stable	**R-45377**^{T} = LMG 26260^T = JCM 19378^T	98
Methyloparacoccus	M. murrellii	NA	pMMO	5.8-9 (6.3-6.9)	20-37 (25-33)	Strain was isolated from pond water Nitrogen fixation	**LMG 27482**^{T} = JCM 19379^T	99
Methyloprofundus	M. sedimenti	0.08	pMMO RuMP, pSC	6-8 (6.5-7.5)	4-26 (23)	Nitrogen fixation	Draft genome **WF1** = BCCM LMG 28393 = ATCC BAA-2619) [Draft genome]	100

Methylosarcina	*M. fibrata* AML-C10	0.08	pMMO, RuMP, pSC	5-9 (5)	10-37 (30)	Grows well at pH 5.0. Isolated from landfill soil	**AML-C10^T** (= ATCC 700909^T = DSM 13736^T) [NZ_ARCU00000000.1]	101
	M. lacus LW14	0.05	pMMO, RuMP, pSC	NA (6.8)	NA (6.8)		**LW14^T** (=ATCC BAA-1047^T = JCM 13284^T) [NZ_AZUN00000000.1]	96
Methylothermus	*M. subterraneus* HTM55	0.3	pMMO, RuMP	5.2-7.5 (6)	37-65 (55-60)	Grows well on methane and methanol. Moderately-acidophilic, thermophilic strain	**HTM55^T** (=JCM 13664^T = DSM 19750^T)	102
Methylovulum	*M. miyakonense* HT12	0.009	pMMO, sMMO, RuMP	6-7.5	5-34 (24)	High sensitivity to NaCl	**HT12^T** (=NBRC 106162^T = DSM 23269^T = ATCC BAA-2070^T) 106162^T = DSM 23269^T = ATCC BAA-2070^c [NZ_AQZU00000000.1]	103
				Group II				
Methylosinus	*M. trichosporium* OB3b	0.12	pMMO, sMMO, SC-EMP, H4MTP, H4FP.	6-8 (6.8)	5-37 (30)	Well-established catalysis for PHB production, epoxidation. Are heat-resistant, form spores	NZ_ADVE00000000.2 **OB3b**, Dr. Stein culture collection (University of Alberta) [NZ_ADVE00000000.2]	79
	Methylosinus sp. LW4	0.12-0.15	pMMO, SC-EMP, H$_4$MTP, H$_4$F	6-8 (6.8)	5-37 (30)	Strain shows very robust growth	PRJNA199154 **LW4**, Dr. Lidstrom culture collections (University of Washington) [PRJNA199154]	96,104

Table 13.1: General summary for publicly available methanotrophic cultures—cont'd

Name	Species*	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	Reference
		Growth rate (h^{-1})	Metabolic pathways	pH	T °C	Other		
Methylocystis	*M. rosea* SV97	NA	pMMO, SC-EMP, H$_4$MTP, H$_4$F	5.5-9.0	4-40 (30)		**SV97**^{T} (=DSM 17261^T=ATCC BAA-1196^T) [NZ_ARCT00000000.1]	74,105
	Methylocystis Rockwell	0.16	pMMO, SC-EMP, H$_4$MTP, H$_4$F	NA (6.8)	NA (30)	Highly tolerant of ammonium, capable of nitrogen fixation	**ATCC 49242** [NZ_AJTV00000000.1]	44,88
	M. parvus OBBp	0.12	pMMO, SC-EMP, H$_4$MTP, H$_4$F	6-8 (6.8)	5-37 (30)	Does not form spores, resistant to desiccation, but not heat. Tested for calcium carbonate precipitation	**OBBP** (NCIMB 11129) [AJTV00000000]	79,106
	Methylocystis sp. SC2	NA	pMMO, SC-EMP, H$_4$MTP, H$_4$F	NA	NA	Has low affinity pMMO	**SC2**, Dr. Liesack culture collection (Max Planck Institute) [HE956757/FO000001 and FO000002]	107
	Methylocystis sp. SB2		pMMO, sMMO, SC-EMP, H$_4$MTP, H$_4$F	6-9 (6.8)	10-30 (30)	Facultative methanotroph with EMP pathway. Low CCE (12-13%)	**SB2**. Dr. Semrau lab culture collection (University of Michigan) [NZ_AYNA00000000]	108
Methylocella	*M. silvestris*	0.02	sMMO, Mxa, SC-GS	4-7.5 (5.5)	4-30 (15-20)	Facultative methanotroph, can use short-chain alkanes	**BL2**^{T} (=DSM 15510^T=NCIMB 13906^T) [NC_011666.1]	109

Methylocapsa	*M. acidiphila* B2	0.03	sMMO, Mxa, SC-GS		3-6 (4.5)	Facultative methanotroph	**B2**^{T} (= DSM 13967^T = NCIMB 13765^T) [NZ_ATYA00000000.1]	110
	M. aurea KYG	0.018	pMMO, Mxa, SC-GS	5.2-7.2	2-33	Facultative methanotroph. Capable of nitrogen fixation	**KYG**^{T} (=DSM 22158^T = VKM B-2544^T) [JQKO00000000.1]	111
Methyloferula	*M. stellata* AR4	0.005	sMMO, Mxa, SC-GS/CBB	3.5-7.2			**AR4**^{T} = DSM 22108^T = LMG 25277^T = VKM B-2543^T [NZ_ARWA00000000.1]	112
			Group III					
Methylacidiphilum	*M. fumariolicum* SolV	0.07-0.08	pMMO, Xox, CBB	0.8-5.8 (2)	40-65 (55)	Grows at very low pH. Capable of nitrogen fixation.	**SolV**. Dr. Op den Camp culture collection (Radboud University Nijmegen) [PRJEA85607]	89,113,114
	M. kamchatkensis Kam1	0.01	pMMO, Xox, CBB	2-5 (3)	40-65 (55)	Thermoacidophilic methanotroph	**Kam1**. culture collection (University of Bergen) [PRJNA169117]*	115
	M. infernorum V4	0.03	pMMO, Xox, CBB	1-6 (2)	50	Thermoacidophilic methanotroph	**V4, Dr**. Dunfield culture collection (University of Calgary) [NC_010794.1]	12
Methylacidimicrobium	*M. cyclopophantes*	0.042	pMMO, Xox, CBB	0.6-6 (1.5-3)	20-49 (44)	Mesophilic acidophilic methanotrophs	**3B**, Dr. Op den Camp culture collection (Radboud University Nijmegen)	116

Table 13.1: General summary for publicly available methanotrophic cultures—cont'd

Name	Species*	Growth parameters					Culture collection ID [Genome Accession Number (NCBI)]	Reference
		Growth rate (h^{-1})	Metabolic pathways	pH	T °C	Other		
	M. tartarophylax	0.035	pMMO, Xox, CBB	0.5-5 (1-3)	20-43 (38)	Mesophilic acidophilic methanotrophs	**4 AC**, Dr. Op den Camp culture collection (Radboud University Nijmegen)	116
	M. fagopyrum	0.013	pMMO, Xox, CBB	0.6-6 (1.5-3)	20-39 (35)	Mesophilic acidophilic methanotrophs	**3C**, Dr. Op den Camp culture collection (Radboud University Nijmegen)	116

**NCBI BioProject Accession number; pSC, partial Serine cycle; NA, data not available.

All of these pathways can be identified in genomes of most methanotrophic Gammaproteobacteria (but S. and Kalyuzhnaya M.G., unpublished). However, it has been shown that most C_1 carbon (about 75%) enters the glycolytic route in two methanotrophic strains, *Methylomicrobium alcaliphilum* 20Z and *Methylomonas* sp. LW13.[47] The FBP variant of the RuMP is the most efficient pathway for C_1 assimilation (Figure 13.3a). It enables the conversion of 3 mol of formaldehyde into 1 mol of a three-carbon compound (assumed to be 2-phosphoglycerate for the convenience of balancing) with the generation of 1 mol NADH and 0.7 mol ATP.[47] In *M. alcaliphilum* 20Z and *Methylomonas* sp. LW13 part of the cell carbon (about 25%) is assimilated via the KDPG variant (Figure 13.3b), which requires 2 mol of ATP and generates 1 mol NADH upon assimilation of 3 mol of formaldehyde into 2-phosphoglycerate.

A number of phosphoketolases (EC 4.1.2.9, Xfp), a key enzyme of the bifidobacterial shunt, have been detected in all sequenced Gammaproteobacteria (but S. and Kalyuzhnaya M.G., unpublished). Their function in methanotrophs is not clear yet, but it could be predicted that methanotrophic phosphoketolases contribute to C_2-compound production (Figure 13.3c) and might play a role in C_1 assimilation and/or acetate production upon oxygen limitation. The biotechnological potential of the Xfp-based synthetic pathways has been highlighted in recent publications by Bogodar et al.[118,119]

Hps/Hpi-driven assimilation seems to be an ideal starting point for sugar-based technologies aiming to expand application to natural gas or methane resources. The key genes of the pathway have been identified in two groups of methanotrophs: Gammaproteobacteria and ANME.[1,52,54] It has been shown that in methanogenic archaea, the enzymes operate in reverse direction to produce R5P needed for nucleic acid synthesis.[120,121] Nothing is known about Hps/Hpi function in anaerobic methane utilizers, except that members of the ANME consortia express the enzymes.[54] It is tempting to speculate that these enzymes play a role in C_1 assimilation in AnMO.

13.4.2 [CH$_2$]- to Biomass Conversion

It has been suggested that direct nonenzymatic condensation of formaldehyde with tetrahydrofolate (H_4F) contributes to methylene-H_4F formation, an entry metabolite for the SC (Figure 13.4).

However, the kinetics of the chemical condensation is quite unfavorable.[69] It is predicted that the assimilation of C_1 units derived from methane or methanol via the SC involves the H_4F-linked transfer and starts from formate.[69] The SC in methanotrophs can be supported by the glyoxylate cycle (GC)[112,122] or the ethylmalonyl-CoA pathway (EMC)[48,123] for glyoxylate regeneration (Figure 13.4).

The biotechnological potential of the EMC pathway is well presented by Alber.[124] All benefits of the EMC conversion could be even further extending by the use of methanotrophs. According to genomic evidence, the SC-EMC pathway seems to be the most common

arrangement of the C_1 assimilation in Alphaproteobacterial methanotrophs.[48,104,123] It has been shown that a significant portion of cell carbon in *Methylosinus thrichosporium* OB3b, a model system, comes from CO_2 (over 60%), suggesting that the EMC pathway plays a significant role in assimilation.[48] The overall balance of assimilation via the H_4F, SC, and EMC pathways is: 1.5 mol of formaldehyde and 1.5 mol of CO_2 to make 1 mol of 2-phosphoglycerate, using 2.5 mol of NADH and 4.5 mol of ATP and generating 0.5 mol of ubiquinol (QH_2).

In some microbes, such as *Methylocella*, *Methylocapsa*, and *Methyloferula*, the SC operates in combination with the glyoxylate shunt.[122,125]

The activity of two enzymes of the SC, serine glyoxylate aminotransferase (EC 2.6.1.45) and hydroxypyruvate reductase (EC 1.1.1.81), have been detected in some Gammaproteobacterial methanotrophs.[117] According to available genomic data, all Gammaproteobacterial methanotrophs possess a complete or partial SC; however, none of them have a glyoxylate regeneration pathway. The functional significance of the pathways in C_1 assimilation is not well established. The incomplete SC cannot function as a main assimilatory route and the pathway most likely contributes to biosynthesis of serine or to the conversion of formate into acetyl-CoA (Figure 13.4).

13.4.3 CO_2 to Biomass Conversion

So far only the Calvin-Benson-Bassham (CBB) cycle has been confirmed by experimental measurements as the main assimilatory route in Verrucomicrobial methanotrophs grown in aerobic/microaerobic environments[113] and NC10-phylum grown in anaerobic systems (Figure 13.3c).[126]

Some members of the Group I (*Methylococcus* and *Methylocaldum*) and Group II (*Methyloferula*) are also capable of CO_2 fixation via the CBB.[77,127,128] The CO_2-fixation reactions in these microbes complement other assimilatory routes, contributing to assimilation, energy balancing, and replenishment of some common intermediates of the pentose-phosphate pathway. The CBB cycle would require nine ATP and six NAD(P)H per three-carbon compound.

13.4.4 Other Possible Assimilation Arrangements

Among other possible arrangements of assimilation in methane-consuming bacteria and/or their metabolic partners are: the reductive acetyl-CoA (Wood-Ljungdahl) pathway, which is predicted for oxygen-independent methanotrophy[54]; the reductive tricarboxylic acid cycle (complete or partial), predicted for some Gammaproteobacterial methanotrophs[45]; and the dicarboxylate/4-hydroxybutyrate, the 3-hydroxypropionate/malyl-CoA, and the 3-hydroxypropionate/4-hydroxybutyrate cycles.[72,73] It remains to be seen whether these pathways are used by natural methanotrophs.

13.5 Selecting the Right Microbe: Available Cultures

This part of the chapter is focused primarily on aerobic methanotrophs, as unfortunately no pure microbial cultures capable of carrying out AnMO are available as laboratory strains. Examples of methanotrophic bacteria that could be considered for industrial applications are shown in Table 13.1.

The summary includes microbial cultures that are currently available from culture collections or laboratory stocks. Some specific parameters of different species incorporated in the description of strain are growth rate, methanotrophic network arrangement, and additional unique phenotypic characteristics. It should be remembered that growth characteristics vary significantly even among one species.

Aerobic methane utilization has been described for Gammaproteobacteria (Group I), Alphaproteobacteria (Group II), and Verrucomicrobia (Group III) and predicted for the Deltaproteobacteria (SAR324-clade, a yet-uncultivated marine bacterium).[129] Only members of Group I, II, and III are currently available in pure culture.

13.5.1 Group I: Gammaproteobacterial Methanotrophs

Gammaproteobacterial methanotrophs represent one order, Methylococcales, that includes three families: Crenotrichaceae, represented by one filamentous bacterium *Crenothrix polyspora* Cohn 1870, yet to be isolated in pure culture[130]; *Methylococcaceae*, which currently includes 14 genera (Table 13.1); and *Methylothermaceae*, which includes three genera.[131] Partial or complete genomes are currently available for cultivated representatives of at least 14 genera, with several additional strains in sequencing pipelines (Table 13.1). So far all characterized Gammaproteobacterial strains demonstrate high activity of the ribulose-monophosphate pathway enzymes (Figure 13.3).

Many members of the class are capable of nitrogen fixation[100,127] and almost all grow equally well on methane and methanol. Most methanotrophs of this group accumulate glycogen (up to 60% of cell biomass) as a carbon-storage compound.[132] There are some reports of PHB accumulation, which should be taken with some caution. The lipid-dye test typically used for the detection of PHB might also stain intracellular membrane compartments (ICMs) formed by many members of Group I. So far, PHB-biosynthesis genes have been found only in the genome of *Methylocaldum szegediense* O-12 (Dr. Khmelenina V.N, personal communication).

Representatives of Group I include acidophilic/acidotolerant,[133,134] alkaliphilic/alkalitolerant,[91–93] halophilic/halotolerant,[85,86,135] and thermophilic/thermotolerant[77,87,102,136] species. Two members of the class, *M. capsulatus* and *Methylomonas* spp. are well-established catalysts for SCP,[2,137,138] carotenoids,[6,7] and exopolysaccharides/glycogen production.[7] The biotechnological potential of members of the group has been broadly

discussed.[4,5,139] It has been shown that Gammaproteobacterial methanotrophs are capable of methane fermentation, which expands the applicability of methane conversion from production of biomass-based products to synthesis of chemicals.[47] Many Group I methanotrophs are capable of producing commercially valuable osmoprotectors, such as ectoine and sucrose.[78,91,140] The *M. alcaliphilum* 20Z is the first nonphototrophic microbe to be investigated for direct conversion of methane into extractable fermentation-ready sucrose.[8]

The drawbacks of the group include unstable growth, spontaneous cell lysis, and sensitivity to ammonia.[88] Some species (such as *Methylomicrobium* and *Methylococcus*) seem to be more robust than others (*Methylomonas* and *Methylobacter*). The obligate methanotrophic nature of all members of the group also constrains modern metabolic engineering and requires the development of novel out-of-the-box approaches for the alteration of metabolite fluxes.

13.5.2 Group II: Alphaproteobacterial Methanotrophs

Methanotrophic Alphaproteobacteria are classified into two families: Beijerinckiaceae (genera *Methylocella*, *Methylocapsa*, and *Methyloferula*) and Methylocystaceae (genera *Methylocystis* and *Methylosinus*) in the order Rhizobiales. A summary of a few strains that show good growth is shown in Table 13.1. The drawbacks of the group include quite slow growth (6-8 h for the fastest cultures) and low CCEs (63-64). The main advantages of the group include high resistance to desiccation, more robust growth in the lab, and much greater metabolic flexibility. Many members of the class are capable of nitrogen fixation and some are facultative methanotrophs, utilizing ethanol, acetate, and short-chain alkanes.[141] Carbon assimilation occurs via the SC (GC variant is typical for Beijerinckiaceae; EMC variant is typical for Methylocystaceae members). Some members of the group show high activities of the Calvin cycle enzyme.[112] Two members of the group, *M. thrichosporium* OB3b and *Methylocystis* spp. have extensively been studied for PHB production,[3,142] epoxidation,[11] methanol,[143] bioleaching,[9] calcium carbonate precipitation,[10] and bioremediation.[15] The ability of *Methylocella silvestris* to use short-chain alkanes further expands its biotechnological potential.[141] The Group II methanotrophs are capable of fermenting methane and excreting acetate, succinate, acetone, 2,3-butanediol, and isopropanol.[144,145] It has been predicted that these products are derived from PHB, but the exact mechanisms and pathways are not yet understood.

13.5.3 Group III: Verrucomicrobial Methanotrophs

Verrucomicrobial methanotrophs are a relatively new clade of methane utilizers, which currently includes three genera: *Methylacidiphilum*,[12,146,147] *Methylacidimicrobium*,[116] and *Methyloacida*.[115] These microbes typically inhabit geothermal and acidic environments, where they thrive at pH 0.5-5 (optima 2) and temperature 30-65°C (optima between 35-50°C). Unlike proteobacterial methanotrophs, which prefer to assimilate carbon from reduced forms

of C_1 carbon, all Group III members are autotrophs and use methane only as a source of energy, burning it all the way to CO_2. Despite all theoretical predictions of low yield and low CCE for CBB conversion, the Verrucomicrobial methanotrophs show exceptionally good growth parameters, comparable to the growth of other methanotrophs (Table 13.1).

Many members are capable of nitrogen fixation[148] and accumulate glycogen as a storage compound.[149] Methanotrophic Verrucomicrobia are the only methanotrophs capable of growth at low pH, relatively high osmolality, and high temperature. All these parameters are ideal for the fermentation of organic acids from methane. The biotechnological applications of the group are limited by the lack of appropriate tools for genetic alterations. As this relatively new area of methanotrophy is quickly developing,[89,115,116,146–149] significant progress can be expected in near future.

13.5.4 NC10

While the NC10-phylum members appear to thrive in anaerobic ecosystems with a substantial input of nitrite, at the metabolic level the microbes resemble canonical aerobic methanotrophs.[150] NC10 is predicted to assimilate carbon via the CBB cycle. The only exception of the methane network in NC10 is the presence of a yet-to-be characterized enzyme for intracellular production of oxygen from NO.[151] Despite the aerobic machinery for methane oxidation, the growth and methane oxidation kinetics of NC10 are well below AnMO-SR, and are similar to other AnMO-DR processes. Lack of pure strains and genetic tools, its requirement for NO_2, and its slow kinetics are obviously downsides of the group. However, the microbes can be a source of novel enzymes, such as its enzymatic system responsible for intracellular oxygen production. Once fully established, its enzymatic system could be incorporated into aerobic microbes to improve mass transfer upon gas fermentation or to increase the rate of oxygen-dependent conversion in other biotechnological applications.

13.5.5 ANME

Lack of pure cultures, slow kinetics, and exceptionally slow growth rates (typically below $0.001\,h^{-1}$) makes the anaerobic methanotrophic communities quite a challenging metabolic platform. Furthermore, anaerobic methanotrophic consortia show the lowest CCE, covering only 0.25-1.3 mol % of the methane oxidized.[54,152] Little to nothing is known about optimal cultivation parameters of ANME, and no genetic strategies for metabolic alterations exist. ANME cocultures have been established in a few laboratories, providing some background data to navigate cultivation attempts.[54,55] Nevertheless, it should be remembered that while it appears to be mission impossible for canonical industrial applications, the AnMO might be a breakthrough in C-H activation.[17] Metagenomic and metatranscriptomic data are available for some bioreactor studies, and those can represent *in silico* resources for synthetic applications and computational simulations for metabolic engineering of C_1 metabolism in alternative hosts.

13.6 Looking for a New Microbe: Isolation Strategies

It is always possible that the ideal microbe for biocatalysis still hides in nature. It is hard to cover all possible isolation strategies, as they are typically directed by the parameters relevant to technology of interest. However, a few general concepts can be outlined. As methanotrophic bacteria are widespread in nature, it is the best to find a natural environment which is similar to required cultivation conditions. Isolation of methanotrophic bacteria typically occurs in three stages: (1) enrichment culture; (2) active mixed culture; and (3) pure culture(s).

13.6.1 Enrichment and Mixed Cultures

The initial enrichment cultures should be set up using conditions close to the environmental setting. It is best to use water samples/extracts directly from the source. The enrichment culture should be set up in closed vials with inoculum (10-50% of the enrichment culture) and methane (10-20% of headspace). If an environmental water sample is not available or limited, key environmental parameters such as pH, temperature, and salinity should be precisely represented. It is also best to keep the concentration of oxygen as close to the environmental setting as possible. If parameters are not known for the isolation of aerobe microbes, the oxygen concentration should be kept at 5-10% (of headspace). It is best to monitor the enrichment process by tracing methane, oxygen, and CO_2 using GC-chromatography. When 20-50% of added methane is consumed, the culture should be transferred into a new vial containing higher amounts of nutrients. The cultivation medium should replicate environmental sample parameters, with an excess of main nutrients, such as nitrate, phosphate, sulfate, copper, and iron. This can be achieved by adding mineral medium (see Table 13.2) or similar media like Medium 632 and 1179 (http://www.dsmz.de) to the filtered water sample/extract from the environment at a 5:100 ratio.

The ratio can be increased to 50:50 and completely replaced with just medium in follow-up cultures. The growth of cultures should be monitored by optical density and gas consumption. This approach should lead to an active mixed culture.

13.6.2 Pure Culture Isolation

While getting an enrichment culture of methanotrophic bacteria is always quite easy, it is not always an easy task to separate a pure culture out of the well established, usually 3-10 species, consortia. A number of tricks have been used for identification and isolation of methanotrophs[153,154]; see also methanotroph.org. In some cases, the interactions between methanotrophs and satellite cultures are quite strong. One of the tricks for the separation of a methanotrophic strain is cultivation on a mixed-culture filtrate. To prepare the filtrate, the mixed culture should be grown to mid-exponential phase. The liquid phase should be separated from the cells by filtration via a 0.2-0.02 μm filter. The filtrate can then be used for serial dilutions. To make solid medium, the filtrate should be mixed with hot sterilized

Table 13.2: An example of growth medium for methanotrophic bacteria isolation and cultivation

Compounds	Amount	Comments
KNO_3	1 g	Autoclave at 121°C for 30 min. Cool. Add phosphate solution
$MgSO_4 \times 7H_2O$	0.2 g	
$CaCl_2 \times 2H_2O$	0.02 g	
Trace solution	1 ml	
Phosphate solution	20 ml	
Distilled deionized water	1 L	
Trace solution	g/L	Autoclave at 121°C for 20 min. After autoclaving the solution becomes pink
Na_2EDTA	5	
$FeSO_4 \times 7H_2O$	2	
$ZnSO_4 \times 7H_2O$	0.3	
$MnCl_2 \times 4H_2O$	0.03	
$CoCl_2 \times 6H_2O$	0.2	
$CuSO_4 \times 5H_2O$	0.6	
$NiCl_2 \times 6H_2O$	0.05	
$Na_2MoO_4 \times 2H_2O$	0.05	
H_3BO_3	0.03	
Phosphate solution	g/L	pH of the solution should be 6.8-7. Autoclave at 121°C for 20 min
KH_2PO_4	5.44	
Na_2HPO_4	5.68	

medium containing 5% agar, at a 5:1 ratio. The isolation of a pure culture can be achieved by serial dilution and/or by spread plating on solid media.

13.7 Concluding Remarks

While aerobic methanotrophs are often seen as the most credible systems for methane biocatalysis, it should be kept in mind that natural microbes typically do not experience selective pressure for high rate/high CCE of methane utilization. In most environments, methanotrophic bacteria are part of a very complex community, and their functions realized under different environmental constrains such as nutrient limitation, competition, synergistic interactions, or predation. Such wild cultures possess specialized metabolic networks (which might rely on activities of other members of the community) and complex signaling mechanisms (which activate in response to high population density), and those might lead to granulation or lysis. Obviously, these factors might impact the performance of the selected strain in "man-made" settings or upon industrial scale-up.

When comparing all known clades of methane utilizers, it is not surprising that Gammaproteobacterial methanotrophs are the winners. They have the highest growth rate and the highest CCE of methane utilization, which is not surprising when their highly efficient arrangement of methane utilization is taken into account. However, both Alphaproteobacteria

and Verrucomicrobia offer some additional advances that keep them on the consideration list: (1) only Verrucomicrobial methanotrophs can tolerate extremely low pH and they are an attractive system for the conversion of methane sources into organic acid and (2) so far only Alphaproteobacterial methanotrophs have shown metabolic flexibility. They are able to use other sources of carbon in addition to C_1 compounds. That makes them a unique system for the manipulation of C_1 machinary for biological production of methanol from methane. In addition, the ability of some members of the group to co-utilize short alkanes has obvious benefits for natural gas conversion. Finally, these microbes have a high flux of carbon into CoA-derivatives, such as acetyl-CoA and crotonyl-CoA.

A number of pathways have been described for anaerobic methane consumption, but none of them can be driven by a single pure culture. The methane oxidation in the NC10 phylum is driven by aerobic MMO, but mechanisms supporting how the cells generate oxygen remain unclear. The slow rate of methane oxidation, the lack of pure cultures, the need for expensive electron acceptors, the generation of toxic by-products (such as H_2S or ammonia), and the need for conceptually new approaches for genetic manipulation restrict the large-scale deployment of anaerobic methane conversion today. Nevertheless, these novel microbes represent a very valuable source of enzymes for the synthetic biology applications.

Acknowledgments

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