



**HAL**  
open science

## Synthetic methylotrophy: past, present, and future

Stephanie Heux, Trygve Brautaset, Julia A. Vorholt, Volker F. Wendisch,  
Jean-Charles Portais

► **To cite this version:**

Stephanie Heux, Trygve Brautaset, Julia A. Vorholt, Volker F. Wendisch, Jean-Charles Portais. Synthetic methylotrophy: past, present, and future. Methane Biocatalysis: Paving the Way to Sustainability, Editions Springer, 2018, 978-3-319-74865-8. hal-02154351

**HAL Id: hal-02154351**

**<https://hal.science/hal-02154351>**

Submitted on 19 Jan 2023

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



# Synthetic Methylotrophy: Past, Present, and Future

# 9

Stephanie Heux, Trygve Brautaset, Julia A. Vorholt,  
Volker F. Wendisch, and Jean Charles Portais

## 9.1 Natural Methylotrophy

### 9.1.1 Definition and Phylogenetic Assignment of Methylotrophs

Methylotrophy is the capacity of certain microorganisms (mostly bacteria and yeasts) to use reduced molecules without C–C bond (also referred to as one-carbon (C1) compounds) as their sole source of carbon and energy. Phylogenetically, natural methylotrophs belong to a rather small number of genera. The majority of methylotrophic bacteria are members of the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria* (e.g., *Methylobacterium*, *Methylobacillus*, and *Methylococcus*); alternatively, they can be found within the Gram-positive and *Verrucomicrobia* (e.g., *Bacillus* and *Methylacidiphilum*, respectively) groups (Kolb 2009; Lidstrom 2006). The eukaryotic methylotrophs include the yeasts *Candida*, *Pichia*, and some genera that were recently separated from *Pichia* (i.e., *Ogataea*, *Kuraishia*, and *Komagataella*) (Yurimoto et al. 2011).

---

S. Heux (✉) · J. C. Portais

LISBP, Université de Toulouse, CNRS, INRA, INSA, Toulouse, France

e-mail: [heux@insa-toulouse.fr](mailto:heux@insa-toulouse.fr); [portais@insa-toulouse.fr](mailto:portais@insa-toulouse.fr)

T. Brautaset

Department of Biotechnology, Norwegian University of Science and Technology (NTNU),  
Trondheim, Norway

e-mail: [trygve.brautaset@ntnu.no](mailto:trygve.brautaset@ntnu.no)

J. A. Vorholt

ETH Zurich, Zurich, Switzerland

e-mail: [jvorholt@ethz.ch](mailto:jvorholt@ethz.ch)

V. F. Wendisch

Genetics of Prokaryotes, Faculty of Biology & CeBiTec, Bielefeld University, Bielefeld, Germany

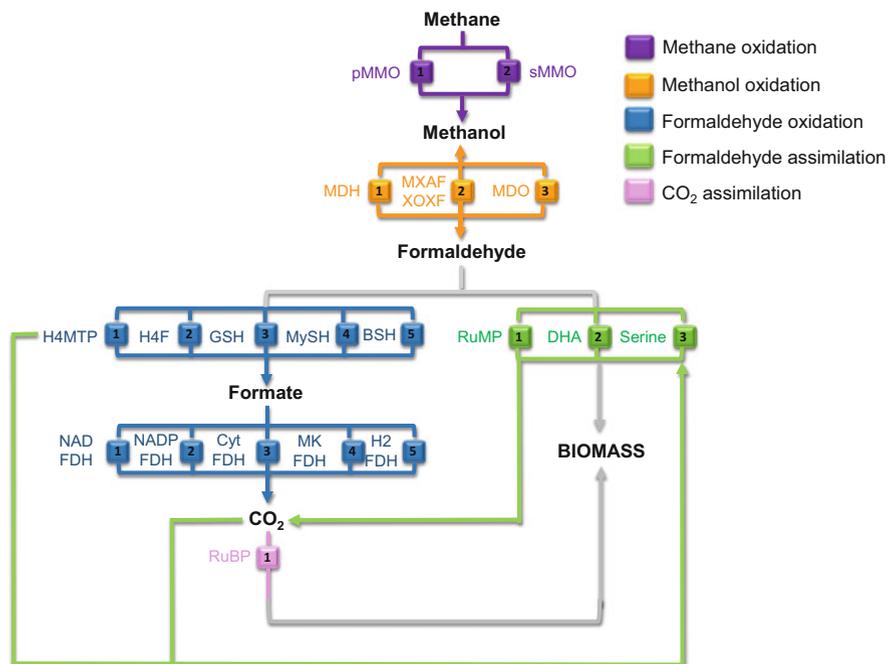
e-mail: [volker.wendisch@uni-bielefeld.de](mailto:volker.wendisch@uni-bielefeld.de)

### 9.1.2 C1 Compounds Utilized by Methylootrophs

The C1 compounds used by natural methylootrophs include methane and methanol, and also methylamines (di-, tri-, and tetramethylamine), formate, formamide, chloromethane, and dichloromethane. While bacterial methylootrophs typically are capable of growing on a variety of C1 compounds—with the exception of methanotrophs, which are mostly dedicated to methane conversion—eukaryotic methylootrophs are restricted to growth with methanol as C1 source. The utilization of methane, methanol, and other C1 compounds as sole carbon and energy source requires two major biochemical processes. First, it involves dissimilation pathways coupling the oxidation of C1 substrate with energy conservation process to fulfill the energetics requirement for growth. Second, it requires assimilation pathways to incorporate the carbon atom into cell material, which is achieved by the fixation of either free formaldehyde, methylene tetrahydrofolate (methylene- $H_4F$ ), or  $CO_2$  in known methylootrophic pathways. For both dissimilation and assimilation processes, a diverse range of alternative enzymes or pathway variants and combinations thereof exist in nature (for a review, see Chistoserdova 2011). In this chapter, we focus on methane- and methanol-utilizing aerobic bacteria and fungal (yeast) methylootrophs. The pathways involved in this metabolism can be categorized as follows: (1) oxidation of methane to methanol, (2) oxidation of methanol to formaldehyde, (3) oxidation of formaldehyde to  $CO_2$ , and (4) assimilation of C1 compounds (see more details in Fig. 9.1 and below).

### 9.1.3 Pathways of C1 Oxidation

Methane oxidation is catalyzed by the enzyme methane monooxygenase (MMO). In the MMO reaction, one oxygen atom is incorporated into methane to form methanol, and the other oxygen atom is released as water, requiring the input of two electrons and two protons. Two forms of MMO exist, a cytoplasmic or soluble form (sMMO) and a membrane-embedded form (also named particulate form, pMMO) (Hakemian and Rosenzweig 2007). sMMO occurs in a smaller group of methanotrophs and consists of three subunits, a hydroxylase (MMOH), a reductase (MMOR), and a regulator (MMOB). The hydroxylase itself consists of the three subunits  $\alpha$ ,  $\beta$ , and  $\gamma$  (Merckx et al. 2001). pMMO is produced by almost all methanotrophs (with few known exceptions; Dedysh et al. 2015) and is composed of three subunits encoded by the genes *pmoA*, *pmoB*, and *pmoC* (Balasubramanian et al. 2010). The two MMO forms exhibit significantly different catalytic characteristics, including substrate specificity, enzyme stability, specific activity, and susceptibility to inhibitors (Murrell et al. 2000). For example, sMMO has a much broader substrate range compared with pMMO, which includes long-chain alkanes or aromatic molecules (Murrell et al. 2000). In terms of reducing power, the sMMO uses NADH, while the physiological electron donor of the pMMO is still not known (Kalyuzhnaya et al. 2015). The active sites of sMMO and pMMO also contain different metal ions, i.e.,



**Fig. 9.1** A simplified diagram showing the major metabolic modules involved in methylotrophy. See section “Natural Methylophony” for more details

iron and copper, respectively (Murrell et al. 2000). pMMO is expressed under a high copper concentration (Murrell et al. 2000).

Methanol is oxidized to formaldehyde by methanol dehydrogenases. Gram-negative methylotrophs possess a periplasmic cytochrome *c*-dependent methanol dehydrogenase, the catalytic subunit of which is encoded by *mxAF* or *xoxF*, respectively. The enzymes contain a pyrroloquinoline quinone (PQQ) cofactor (Ghosh et al. 1994) and Ca<sup>2+</sup> (MXAF) or rare earth elements (XOXF) (Hibi et al. 2011; Pol et al. 2014). Thermophilic Gram-positive methylotrophs of the genus *Bacillus* use a cytoplasmic NAD-dependent methanol dehydrogenase (MDH) (Arfman et al. 1989) or a nicotinoprotein methanol dehydrogenase (MDO) with a bound NAD(P) cofactor that uses an unknown electron acceptor for reduction (Bystrykh et al. 1993). In methylotrophic yeasts, methanol is oxidized by the enzyme alcohol oxidase (AOD) in a reaction that uses oxygen as an electron acceptor and produces not only one but two reactive species, i.e., formaldehyde and hydrogen peroxide (detoxified by catalase) (Yurimoto et al. 2011).

A number of paralogous pathways exist for the efficient oxidation of the cell-toxic intermediate formaldehyde to CO<sub>2</sub> (dissimilation), sometimes even within one organism (Vorholt 2002). They comprise linear cofactor-dependent pathways, such as the tetrahydromethanopterin (H<sub>4</sub>MPT) or tetrahydrofolate (H<sub>4</sub>F)-dependent pathway, which are widespread among methylotrophic Proteobacteria (Vorholt

2002). Other linear oxidation pathways are dependent on thiol compounds, such as glutathione (GSH, *Paracoccus denitrificans*), mycothiol (MySH, Gram-positive methylotrophs), and bacillithiol (BSH, *B. methanolicus*) (Vorholt 2002; Muller et al., 2015a). All these pathways use formate as an intermediate which is then oxidized to CO<sub>2</sub> via formate dehydrogenases (FDHs). A large number of FDHs exist that differ by the cofactor they use (i.e., NAD, NADP, cytochrome, menaquinone, H<sub>2</sub>) (Chistoserdova 2011). A cyclic dissimilatory ribulose monophosphate pathway exists in other Gram-negative *Proteobacteria* (e.g., *M. flagellatus*) and in thermophilic bacilli for oxidation of formaldehyde to CO<sub>2</sub> (Anthony 1991; Chistoserdova 2011; Muller et al., 2015a). It overlaps with the ribulose monophosphate pathway for C1 assimilation (see section below) with the additional participation of 6-phosphogluconate dehydrogenase.

### 9.1.4 Pathways of C1 Assimilation

The assimilation of C1 precursors in methylotrophs occurs either from formaldehyde or from CO<sub>2</sub>. There are four different pathways by which aerobic methylotrophs assimilate carbon into cell material and each has at least two potential variants. One route, the serine cycle, involves carboxylic acids and amino acids as intermediates, whereas the other three routes involve (phosphorylated) carbohydrate intermediates (the ribulose biphosphate (RuBP) pathway, the ribulose monophosphate (RuMP) pathway, and the dihydroxyacetone (DHA) pathway).

The serine cycle for C1 assimilation occurs in *Alphaproteobacteria* (e.g., *M. extorquens*). In these bacteria, C1 units are assimilated in the form of formaldehyde via methylene-H<sub>4</sub>F catalyzed by serine transhydroxymethylase yielding serine from glycine. In addition, CO<sub>2</sub> is fixed in the serine cycle via a carboxylase (Quayle 1972). The assimilation of C1 units via the serine cycle requires regeneration of glyoxylate and subsequently glycine from acetyl-CoA. This is achieved either by the glyoxylate cycle in isocitrate lyase containing methylotrophs (ICL<sup>+</sup> variant) (Chistoserdova 2011) or by the ethylmalonyl-CoA pathway (EMCP) in serine cycle methylotrophs that lack ICL (i.e., ICL<sup>-</sup> variant) (Erb et al. 2007; Peyraud et al. 2009).

The RuBP pathway, also known as the Calvin-Benson-Bassham (CBB) cycle, occurs in a few known methylotrophs (e.g., *Paracoccus denitrificans*) (Chistoserdova 2011). In these organisms, the C1 substrate is first oxidized to CO<sub>2</sub>, which can be further assimilated in the CBB cycle, as in classical autotrophic organisms. The fixation part of this pathway involves the carboxylation of ribulose-1,5-bisphosphate (RuBP) to 3-phosphoglycerate (3PG) in a reaction catalyzed by ribulose biphosphate carboxylase. The C3 compounds are then used either as precursors of cell biomass or by a mechanism of carbon skeleton recombination to refill the RuBP pool. In total three molecules of CO<sub>2</sub> are processed into a single molecule of 3PG (Bassham et al. 1950).

In principle, the RuMP pathway is similar to the RuBP pathway, except that the C1 is assimilated at the level of formaldehyde. The RuMP pathway operates in

*Betaproteobacteria* and *Gammaproteobacteria* as well as in Gram-positive bacteria (e.g., *B. methanolicus*). Through the RuMP pathway, essentially all the carbon required for biomass formation is assimilated from formaldehyde in a reaction catalyzed by 3-hexulose-phosphate synthase (HPS) that condenses formaldehyde and Ru5P into hexulose-6-phosphate (H6P), which subsequently is isomerized to fructose-6-phosphate (F6P) by 6-phospho-3-hexuloisomerase (PHI) (Anthony 1991). F6P is then further cleaved either to glyceraldehyde-phosphate and dihydroxyacetone-phosphate by fructose-bisphosphate aldolase (FBA variant) or to glyceraldehyde-3-phosphate and pyruvate by keto-hydroxyglutarate-aldolase (KDPG aldolase variant). Regeneration of the initial acceptor Ru5P is achieved via a sequence of reactions for which several variants exist, e.g., involving either transaldolase (the transaldolase variant) or sedoheptulose bisphosphatase (the SBPase variant). The C3 compounds generated in these pathways are further glycolytically converted into pyruvate. Finally, this fixation process allows the formation of one molecule of pyruvate from three molecules of formaldehyde (Anthony 1991).

The DHA pathway, which is also called the xylulose-monophosphate (XuMP) pathway, occurs in methylotrophic yeasts growing on methanol. The DHA pathway is similar to the RuBP and RuMP pathways in principle. The C1 unit (formaldehyde in this case) is condensed with a phosphorylated pentose, xylulose 5-phosphate (Xu5P), catalyzed by dihydroxyacetone synthase. The products of the reaction from one C5 sugar are a triose phosphate (glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone) (Anthony 1991). For every molecule of GAP that is incorporated into biomass, three molecules of formaldehyde are fixed, generating three molecules of DHA and consuming three molecules of Xu5P. Recycling Xu5P is completed through molecular rearrangements similar to the reactions of the pentose phosphate pathway (PPP) (Anthony 1991; Russmayer et al. 2015).

---

## 9.2 Synthetic Methylootrophy

### 9.2.1 Concept

The concept of synthetic methylotrophy is the application of a synthetic biology approach to integrate methylotrophy into nonnative methylotrophic production hosts. Integrating methylotrophy into an established production host can thus provide access to methane or methanol as raw material by benefiting from already established production pathways. In this approach, methylotrophic microorganisms are used as benchmark and serve as donor for the systematic engineering of methylotrophy into biotechnology-relevant non-methylotrophic hosts, e.g., *Escherichia coli* or *Corynebacterium glutamicum*.

## 9.2.2 Interest

The interest in bioprocess technology using synthetic methylotrophy is growing both in academia and private industry for the following main reasons:

*1. Methane and methanol are attractive raw material for biotechnology* There is a high societal need for a sustainable production of key chemistry, food, and healthcare compounds. Biological structures such as microbial cell factories are logical production systems, and they use sugars and other food-derived raw materials as substrates. This leads to an unwanted competition with the supply of food, and consequently nutrition prices are rising worldwide. The feasibility of a “methane and methanol economy” was already proposed (Kalyuzhnaya et al. 2015; Olah 2013). Methane and methanol are considered as the next-generation carbon feedstock because both are nonfood raw material and occur abundantly throughout nature. Worldwide, the amount of natural gas is estimated to 204 trillion m<sup>3</sup> (Haynes and Gonzalez 2014). This natural gas consists primarily of methane (CH<sub>4</sub>), and the most important application of natural gas is the conversion to synthesis gas (i.e., syngas), a mixture of CO and H<sub>2</sub> obtained by incomplete combustion. Methane is the second most important greenhouse gas; therefore, exploiting it as raw resource in biotechnology would fight global warming right at the source. Like natural gas or biogas, methane is an economic source of carbon for (bio)chemical synthesis (Kalyuzhnaya et al. 2015). This gas is also an important precursor for the synthesis of methanol. Both methane and methanol can be made from any renewable and sustainable resource that can be, respectively, anaerobically digested into biogas (mixture of methane and CO<sub>2</sub>) or that can be converted first into syngas (Methanol Institute 1989; Mes et al. 2003). This is the case for biomass, agricultural and timber waste, solid municipal waste, landfill gas, industrial waste, and a number of other feedstocks. Such “bio-methanol” can also be produced from CO<sub>2</sub> allowing (bio) chemical recycling of this compound.

*2. Biological conversion of methane and methanol provides access to the manufacture of value-added and even novel products so far difficult to produce or even inaccessible to the chemical conversion of methane/methanol* Methane and methanol are currently chemically transformed to compounds used for the manufacturing of materials (e.g., LED and LCD devices), energy (e.g., gas used primarily for cooking and heating applications), fuels, and fertilizers. Microbial conversion of methane and methanol allows the economic bioproduction of a large industrial product portfolio including fine, platform, and commodity chemicals for health and nutrition (Becker and Wittmann 2015).

*3. Potential and limitations of natural methylotrophs* The most straightforward approach for achieving biological conversion of methane/methanol is to employ natural methylotrophs, and to date a few methane and methanol-based products have been made at the commercial scales. The most successful examples are the production of single-cell protein (SCP) (Ekeroth and Villadsen 1991; Matelbs and

Tannenbaum 1968) by methylotrophic bacteria and the production of recombinant proteins such as enzymes, antibodies, cytokines, plasma proteins, and hormones by methylotrophic yeast (Ahmad et al. 2014). Natural methylotrophs were also used to produce commercially relevant chemicals and enzymes (Selvakumar et al. 2008; Strong et al. 2015; Tani 1991; Clomburg et al. 2017; Pfeifenschneider et al. 2017). PHB production from methane is currently being explored by a number of companies in the USA, Russia, and India (Kalyuzhnaya et al. 2015). Recently, methylotrophs have been genetically engineered for the production of value-added compounds that are not synthesized by native metabolism. One example is the engineering of *B. methanolicus* for the production of the diamine cadaverine and for the production of the  $\gamma$ -aminobutyric acid (GABA), which finds application in the bioplastics industry (Naerdal et al. 2015; Irla et al. 2016). Despite significant scientific and technological progress (de la Torre et al. 2015; Gilman et al. 2015), this approach has been limited mainly by our current understanding of cellular metabolism and physiology that is still incomplete and by poorly developed genetic tools for most bacterial methylotrophs (Chung et al. 2010; Schrader et al. 2009).

*4. Industrial production organisms are ideal hosts for synthetic methylotrophy but require integration of methylotrophic pathways into their metabolism* Establishing an orthogonal methylotrophy module for embedding into the metabolism of a particular production host species would provide the opportunity to tap on the full potential of that host species with regard to value-added compounds produced by it. Indeed for all of them, a methanol-based production process can be envisioned (Müller et al., 2015b; Whitaker et al. 2015; Witthoff et al. 2015). *E. coli*, *C. glutamicum*, and *Saccharomyces cerevisiae* are the host cells that provide the most flexible, biologically well-understood, genetically tractable starting point for further engineering. The molecular toolbox for these microorganisms is large; a range of tools and techniques has been developed to iteratively construct and evaluate modified derivatives of these strains. In addition, their product portfolios range from classical products such as amino acids to biofuels and innovative compounds used as building blocks for the chemical synthesis of chemical polymers and pharmaceuticals (Becker and Wittmann 2015).

*5. Synthetic methylotrophy represents a major scientific challenge combining systems and synthetic biology* Methylotrophy is a complex and carefully regulated process where efficient C1 oxidation has to be balanced with an equally efficient C1 assimilation to ensure cell growth and at the same time avoid accumulation of the cell-toxic intermediate formaldehyde. As discussed here, design and engineering of synthetic methylotrophy is a top-down strategy where components and modules representing several interlinked metabolic pathways have to be functionally integrated into host metabolism. This is a complex task, taking biochemical, regulatory, energetic, and stoichiometric aspects of cell metabolism into consideration, and thus combines cutting-edge competences and technologies in systems biology and synthetic biology.

### 9.3 Design of Synthetic Methylootrophs

Methylootrophy has evolved independently several times since a diverse range of alternative enzymes or pathways and of various combinations of these systems exists in nature. Taking into account all this natural biodiversity at each metabolic stage and the potential for creating new enzyme properties, there is a multitude of routes that can be designed to introduce methane or methanol assimilation into non-methylootrophic hosts.

A number of biochemical and practical considerations have to be taken into account for the design of synthetic methylootrophic pathways. For instance, to establish methane oxidation, introducing sMMO rather than pMMO is easier, because despite being the most common enzyme, the latter one is poorly characterized due to the analytical difficulties to investigate membrane-bound catalysts (Hakemian and Rosenzweig 2007). The physiological source of the electron donor in the pMMO reaction is also still not yet resolved (Kalyuzhnaya et al. 2015), adding some difficulties to efficiently engineer host metabolism with this enzyme. Similarly, the simplest way to engineer organisms for methanol oxidation is to introduce NAD-dependent methanol dehydrogenase since many host reactions can recycle the NADH generated in the MDH reaction. The introduction of PQQ-dependent methanol dehydrogenase (i.e., MXAF and XOXF) is more challenging because PQQ is not as universal as NAD for redox reactions, and hence PQQ recycling is likely to be limited in the host. Moreover, some target organisms, like *E. coli*, cannot natively synthesize PQQ and hence should be further engineered to functionally express PQQ biosynthesis, which is a complex process (Muller et al. 2015b). On the other hand, AOD is also challenging as it produces hydrogen peroxide, which is highly toxic for most of the hosts.

The above considerations can be extended to the other steps of methylootrophy metabolism and emphasize the various levels of biochemical and metabolic complexity to engineer synthetic methylootrophs. They also stress the necessity to consider engineering strategies in a system-wide perspective. To cope with this complexity and to identify the best combination of genes to be introduced into a given non-methylootrophic host, computational tools enabling the *in silico* design of metabolic pathways can be applied (Carbonell et al. 2016; Medema et al. 2012; Vieira et al. 2014). Such computational analyses help to predict and select the best combinations of enzymes and pathways to introduce in the host, as well as the optimization of the host metabolism according to associated metabolic constraints, e.g., cofactor and C1 acceptor regeneration, transport, formaldehyde toxicity, etc. Using this approach, the introduction of MDH together with two reactions from the RuMP pathway (HPS and PHI) was identified as the “best combination” to achieve the highest theoretical growth rate on pure methanol in *E. coli* (Muller et al. 2015b). However, other feasible pathway combinations are conceivable as well.

The choice of which assimilatory pathway to introduce in host organisms will firstly depend on the number of genes to be introduced. For instance, only two heterologous proteins (i.e., HPS and PHI) are theoretically needed to establish a complete RuMP cycle in *E. coli*, while four heterologous proteins (i.e., serine-

glyoxylate aminotransferase serine, glycerate 2-kinase, malate thiokinase, and malyl-coenzyme A lyase) are needed to establish a complete serine cycle. Secondly, it will depend on the desired end product, since high-flux intermediates are different in the diverse pathways. For instance, the assimilatory RuMP pathway will be chosen for the production of sugar-phosphate intermediates, while the serine cycle can be exploited for the production of dicarboxylic acids (Kalyuzhnaya et al. 2015).

Finally, with the advances in synthetic biology, completely novel synthetic metabolic pathways can be created by combining retrosynthesis-based approaches to generate de novo metabolic pathways (Hadadi et al. 2016; Jeffryes et al. 2015; Planson et al. 2012; Schwander et al. 2016) with computational design of enzymes to encode the novel enzymatic steps (Siegel et al. 2015; Erb et al., 2017).

---

## 9.4 Progress in Establishing Synthetic Methylophony

### 9.4.1 Synthetic Methane Utilizer

The first attempts to engineer methane utilization in a nonnative host were performed in *E. coli*. In 1992, West et al. reported on the expression of the sMMO genes from *M. capsulatus* in *E. coli*. They demonstrated functional activity of MMOR and MMOB in the recombinant *E. coli*, but did not show activity of the complete sMMO enzyme complex (West et al. 1992). Later attempts to heterologously express the MMOH gene of sMMO in methanotrophs that only contain pMMO did not successfully reconstitute MMO activity (Lloyd et al. 1999; Murrell 2002; Wood 2002). Concerning pMMO, there was little or no activity when expressed in different hosts including *E. coli* and *Rhodococcus erythropolis*, most probably due to incorrect protein folding (Gou et al. 2006; Balasubramanian et al. 2010) (see Table 9.1).

### 9.4.2 Synthetic Methanol Utilizer

To date, engineering methanol utilization in biotechnologically relevant microbes has been described for *E. coli*, *C. glutamicum*, *Pseudomonas putida*, and *S. cerevisiae* (Dai et al. 2017; Koopman et al. 2009; Lessmeier et al. 2015; Muller et al. 2015b; Orita et al. 2007; Whitaker et al. 2017; Witthoff et al. 2015; Wendisch et al. 2016). These synthetic biology works have in common that they rely on the previously mentioned combination composed by MDH, HPS, and PHI (see Table 9.1).

Synthetic methylophony was described in *E. coli* (Muller et al. 2015b). In this work, expression of the RuMP genes from *B. methanolicus* along with MDH from *B. methanolicus* led to 40% incorporation of  $^{13}\text{C}$ -methanol into central metabolites (Muller et al. 2015b). Another study described a similar approach to engineer *E. coli* with comparable success using MDH from *Bacillus stearothermophilus* (Whitaker et al. 2017). In both works, the metabolites generated from  $^{13}\text{C}$ -methanol were not 100% labeled, indicating that methanol alone cannot supply all carbon atoms for molecular buildup and in consequence does not allow pure methylophony growth.

**Table 9.1** List of hosts with implemented genes for enzymes involved in methane and methanol utilization

Nonnative host	MMO	MDH	HPS	PHI	References
<i>E. coli</i>	<i>smmo</i> operon (mca)	×	×	×	West et al. (1992)
Methanotrophs containing only pMMO	<i>smmo</i> (mca and mtri)	×	×	×	Lloyd et al. (1999), Murrell (2002), Wood (2002)
Rhodococcus erythropolis	pmmo gene cluster (mca)	×	×	×	Gou et al. (2006)
<i>E. coli</i>	pmmo gene cluster (mtri)	×	×	×	Balasubramanian et al. (2010)
Cell-free system	×	<i>mdh</i> (bmet)	<i>hps</i> (mca)	<i>phi</i> (mfa)	Burgard et al. (2014)
<i>E. coli</i> deleted for native formaldehyde oxidation pathway	×	<i>mdh2</i> (bmet)	<i>hps</i> (bmet)	<i>phi</i> (bmet)	Brautaset et al. (2013)
	×	<i>mdh</i> (bste)	<i>hps</i> (mgas)	<i>phi</i> (mgas)	Papoutsakis et al. (2015)
	×	<i>mdh</i> (bste)	<i>hps</i> (bmet)	<i>phi</i> (bmet)	Whitaker et al. (2017)
	×	<i>mdh3</i> (bmet)	<i>hps</i> (mgas)	<i>phi</i> (mgas)	Price et al. (2016)
<i>E. coli</i>	×	<i>mdh2</i> (bmet)	<i>hps</i> (bmet)	<i>phi</i> (bmet)	Muller et al. (2015b)
<i>C. glutamicum</i> deleted for native formaldehyde oxidation pathway	×	<i>mdh2</i> (bmet)	<i>hps</i> (bmet)	<i>phi</i> (bmet)	Brautaset et al. (2013)
	×	<i>mdh</i> (bmet)	<i>hxlA</i> (bsu)	<i>hxlB</i> (bsu)	Lessmeier et al. (2015), Witthoff et al. (2015)
<i>B. subtilis</i> overexpressing genes encoding for Ru5P regeneration enzymes	×	<i>mdh3</i> (bmet)	<i>hxlA</i> (bsu)	<i>hxlB</i> (bsu)	Brautaset et al. (2013)
<i>P. putida</i>	×	×	<i>hps</i> (bbri)	<i>phi</i> (bbri)	Koopman et al. (2009)
<i>S. cerevisiae</i>	×	<i>mdh</i> (bmet)	<i>hxlA</i> (bsu)	<i>hxlB</i> (bsu)	Dai et al. (2017)

Abbreviations: MMO = methane monooxygenase, MDH = NAD-dependent methanol dehydrogenase, HPS = 3-hexulose-phosphate synthase, PHI = 6-phospho-3-hexuloisomerase, mca = *Methylococcus capsulatus*, mtri = *Methylosinus trichosporium*, bmet = *Bacillus methanolicus*, bste = *Bacillus, stearothermophilus*, mgas = *Mycobacterium gastris*, mfa = *Methylobacillus flagellatus*, bsu = *Bacillus subtilis*, bbri = *Bacillus brevis*

However, the central metabolites analyzed showed labeling of multiple carbons (Muller et al. 2015b; Whitaker et al. 2017). This can exclusively be explained by metabolites passing through the RuMP cycle several times demonstrating the in vivo functionality of the RuMP cycle. Molecules having incorporated carbon atoms from methanol also appeared in biosynthetic pathways, e.g., for cell wall biosynthesis (Muller et al. 2015b) and in several proteinogenic amino acids (Whitaker et al. 2017). Higher biomass levels were obtained when methylophony *E. coli* cells were grown on yeast extract + methanol compared to growth on yeast extract alone, also suggesting a contribution of methanol to biomass production. However, only about 0.3 g/L methanol were utilized in the medium containing 1 g/L yeast extract increasing the OD from about 0.7 to about 0.9, while 1.6 g/L methanol remained untouched (Whitaker et al. 2017). Finally incorporating the pathway to synthesize the flavanone naringenin in the former engineered *E. coli* strain allowed in vivo  $^{13}\text{C}$ -labeling of a specialty chemical from  $^{13}\text{C}$ -methanol. However, no net formation of naringenin from methanol was demonstrated (Whitaker et al. 2017). In another approach, *hps* and *phi* genes from *Mycobacterium gastri* MB19 or other methylophony were heterologously synthesized as fusion protein in *E. coli* and exhibited similar or even superior activity in vitro and in vivo (Orita et al. 2007; Muller et al., 2015b).

In *C. glutamicum*, methanol utilization has been achieved by expressing MDH from *B. methanolicus* together with *hxlA* (3-hexulose-phosphate synthase) and *hxlB* (6-phospho-3-hexuloisomerase) from *B. subtilis* (Lessmeier et al. 2015; Witthoff et al. 2015). In the resulting strains, the incorporation of  $^{13}\text{C}$ -label from  $^{13}\text{C}$ -methanol into central metabolites was detected, and molecules were labeled at more than one atom carbon position. This indicates the in vivo operation of the synthetic methanol utilization pathway and demonstrates that the RuMP pathway is indeed “cycling” (Lessmeier et al. 2015; Witthoff et al. 2015). Applying this strategy to a cadaverine-producing strain resulted in  $^{13}\text{C}$ -labeling of cadaverine from  $^{13}\text{C}$ -methanol. Thus, the nonnatural carbon source methanol was converted to the nonnatural product cadaverine by recombinant *C. glutamicum* to some extent; however, net biomass formation from methanol was not observed (Lessmeier et al. 2015).

The solvent-tolerant bacterium *P. putida* was engineered to utilize methanol and formaldehyde as auxiliary substrates by introducing the *hps* and *phi* genes from *Bacillus brevis* (Koopman et al. 2009). In chemostat cultures feeding formaldehyde as co-substrate to glucose, the *hps*- and *phi*-expressing strain showed a two times higher biomass yield compared to the control strain. Furthermore, the *hps*- and *phi*-expressing strain was also able to grow when replacing formaldehyde with methanol, while the control strain did not reach steady state under these conditions. This is probably due the presence in *P. putida* of an endogenous methanol-oxidizing activity which may result from a side activity of a broad-specificity alcohol dehydrogenase (Koopman et al. 2009; Yurimoto et al. 2009). However, authors did not show any evidence that a functional RuMP was operating in vivo.

For the first time, methanol utilization has been described in *Saccharomyces cerevisiae* (Dai et al. 2017). In this study, methanol metabolic pathway originating

from both prokaryotic and eukaryotic methylotrophs was tested. Expression of the *mdh* from *B. methanolicus* MGA3 with the RuMP genes from *B. subtilis* failed to allow methanol consumption and cell growth in a minimal medium containing methanol as the sole carbon source. However, in a *S. cerevisiae* expressing *aod*, together with the XuMP genes from *Pichia pastoris*, a consumption of methanol of 1.04 g/L and 3.13% increase of cell growth were observed. This was further improved when yeast extract was added to the medium (Dai et al. 2017). However, no proof of in vivo functionality of the introduced methanol pathway was shown.

### 9.4.3 Synthetic Methylotrophy in the Patent Literature

With respect to patents, Helman et al. claimed the development of synthetic methanotrophic microorganisms for the production of chemical product. Lynch claimed the development of metabolically engineered strain for the production of acetyl-CoA from methane, methanol, and/or formaldehyde. Coleman et al. claimed the use of metabolically engineered host microorganisms, which utilize methane as the sole carbon source. Burgard et al. claimed the development of nonnaturally occurring microbial organism having a methanol metabolic pathway for producing 1,4-butanediol related thereto. All of them mentioned among others the expression of genes encoding for MMO, MDH, HPS, or PHI; however, none of them provided experimental or quantitative evidence for these claims (Helman et al. 2015; Burgard et al. 2014; Lynch 2014; Coleman et al. 2014). Brautaset et al. described the expression of RuMP pathway enzymes (i.e., HPS and PHI) together with MDH in *E. coli*, *C. glutamicum*, and *B. subtilis*. Except for *B. subtilis*, significant label incorporation was detected when these cells were grown on  $^{13}\text{C}$ -methanol. For engineered *B. subtilis*, label was only detected when five genes encoding for Ru5P regeneration enzymes (i.e., *pfk*, encoding phosphofructokinase; *rpe*, encoding ribulose-5-phosphate 3-epimerase; *tkt*, encoding transketolase; *glpX*, encoding fructose-1,6-bisphosphatase; and *fba*, encoding fructose-1,6-bisphosphate aldolase) were overexpressed in addition to the first set of genes (Brautaset et al. 2013). Papoutsakis et al. observed incorporation of  $^{13}\text{C}$ -label from methanol into central metabolites in *E. coli* strain expressing the *hps* and *phi* gene fusion from *M. gastri* with the MDH from *B. stearotheophilus* (Papoutsakis et al. 2015). See details in Table 9.1.

## 9.5 Perspectives in Engineering Synthetic Methylotrophs

In all the synthetic methanol utilizers described up to now, incorporation of methanol-carbon in biomass, central metabolites, or secreted products could be observed. However, no growth on pure methanol was detected indicating that metabolic bottlenecks remain. Although MDHs are presumably the best candidates for engineering synthetic MeOH utilizers, the kinetics (low affinity toward methanol) and thermodynamic (positive Gibbs energy) properties of these enzymes favor a

low methanol oxidation rate and a higher flux in the reverse direction (i.e., formaldehyde to methanol conversion). In an approach to maintain a low formaldehyde concentration to favor methanol to formaldehyde conversion and to lower formaldehyde-associated toxicity, MDH, HPS, and PHI were assembled into an artificial enzyme complex. By doing so, synthesis of F6P from methanol and RuMP was increased by 50-fold, and production of F6P was further improved twofold *in vitro*, when a “NADH sink” (i.e., a NADH-dependent lactate dehydrogenase) was added to the system (Price et al. 2016). The beneficial effect of enzyme (MDH, HPS, and PHI) assembly was also observed *in vivo* by improving whole-cell methanol consumption rate ninefold (Price et al. 2016). Other promising possibilities to improve methanol oxidation rate include expression of existing or less characterized or engineered or computationally designed enzymes with better kinetics properties.

Another major issue in engineering synthetic methylootrophs is the formaldehyde assimilation, i.e., C1 assimilation. As mentioned above, C1 assimilation in nature involves metabolic cycles and a C1 acceptor that enables the formation of a C–C bond. As already discussed (Muller et al. 2015b), the major challenge in the current synthetic MeOH utilizers seems to be the cyclic mode of operation of the RuMP pathway for biomass production which needs to operate in conjunction with the host’s endogenous central metabolism to allow C1 assimilation into biomass and growth in the presence of methanol as a sole carbon and energy source. This is further supported by the fact that in the absence of the five genes encoding the cyclic operation of the RuMP pathway (i.e., *pfk*, *rpe*, *tkt*, *glpX*, and *fba*), *B. methanolicus* is unable to grow on methanol as sole carbon source (Brautaset et al. 2004). Nonnative hosts possess all the aforementioned genes; hence, establishment of methanol assimilation requires sustaining high flux through the endogenous reactions, which may result in significant constraints on the metabolic network. If the flux is too low, then instead of being assimilated, formaldehyde is dissimilated as CO<sub>2</sub> through the host’s endogenous metabolic routes to avoid accumulation. In methylootrophic *C. glutamicum*, the disruption of the native formaldehyde oxidation pathway improved formaldehyde assimilation (Lessmeier et al. 2015). In contrast, the presence of this pathway in methylootrophic *E. coli* did not affect the formaldehyde production rate (Muller et al. 2015b). Further improvement of formaldehyde assimilation by either boosting C1 acceptor regeneration or avoiding the requirement of the C1 acceptor might help to establish methanol assimilation. Boosting the C1 acceptor regeneration by more balanced production of the utilized enzymes or even by exchanging them with enzymes from natural methylootrophs such as *B. methanolicus* might represent a valuable strategy for overcoming potential problems caused by an insufficient regeneration of the C1 acceptor (Muller et al. 2015b). This has been recently tested and led to improved methanol utilization (Bennett et al. 2018). Alternatively, experimental evolution might be a valuable approach (Muller et al. 2015b) that can be applied alone or in combination with metabolic engineering. In this context, it is interesting to note that recently a “semi-autotrophic” *E. coli* strain was generated in which energy production is decoupled from C1 fixation. The resulting evolved *E. coli* strain is able to convert CO<sub>2</sub> into

sugars and other biomass precursors via a nonnative carbon fixation machinery. To do so, Antonovsky et al. used a strain where the C1 fixation module (CBB cycle) was separated from energy production with the latter being furnished by feeding pyruvate (Antonovsky et al. 2016). Expressing a pathway that does not require recycling of the C1 acceptor might also be a promising strategy and can be achieved by expressing the two genes encoded for MDH and formolase (FLS) (Nguyen et al. 2016). FLS is an enzyme designed computationally from the benzaldehyde lyase, which converts three molecules of formaldehyde into one molecule of DHA (Siegel et al. 2015). In this pathway, optimal operation of FLS is required to avoid formaldehyde accumulation since here three molecules of formaldehyde are condensed instead of one in the RuMP pathway (Bar-Even 2016; Tai and Zhang 2015). Furthermore, suboptimal operation of the FLS reaction only leads to condensation of two molecules of formaldehyde-yielding glycolate, which may not be converted further or may even be toxic. The formolase-based synthetic metabolic pathway has been experimentally tested on formate; further improvements will be required to allow growth on formate/formaldehyde.

Finally, with regard to methane utilization, the heterologous MMO expression remains an obstacle for the reconstruction of a methane assimilation pathway in nonnative hosts. Thus, the development of MMO enzymes that can work properly in nonnative hosts is a prerequisite to obtain synthetic methane utilizer. This will require the molecular engineering of MMO as well as better understanding of the structure and catalytic mechanism of MMO (Strong et al. 2015; Nguyen et al. 2016). Another important issue will be the regeneration of the reducing power NAD(P)H, i.e., required for the oxidation of methane to methanol. This problem can be ameliorated if oxidation of methanol to formaldehyde is made by a NAD(P)-dependent methanol dehydrogenase yielding reduced redox cofactors NAD(P). However, this high energy loss may limit the efficiency of the biological conversion of methane to chemicals and fuels (Conrado and Gonzalez 2014). More efficient activation strategies to capture energy from methane to methanol oxidation will thus be required. This can be achieved by the expression of existing or less characterized or engineered enzymes and by the optimization of the process (e.g., increase methane solubility or reduce process heat loss) (Conrado and Gonzalez 2014).

Synthetic methylotrophy is still in its infancy, and many challenges remain to fully exploit the potential of C1 compounds for valuable and cost-effective biotechnology processes. However, due to the significant progress that has been made in the recent years both in the comprehensive understanding of methylotrophy and in the development of synthetic biology concepts and tools, there is no doubt that these challenges will be addressed in the near future, opening up new opportunities for biotechnology in a broad range of markets.

---

## References

- Ahmad M, Hirz M, Pichler H, Schwab H (2014) Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. *Appl Microbiol Biotechnol* 98(12):5301–5317. <https://doi.org/10.1007/s00253-014-5732-5>

- Anthony C (1991) Chapter 4 – Assimilation of carbon by methylothrophs. In: Israel Goldberg, J. Stefan Rokem, Biology of methylothrophs. Butterworth-Heinemann, Oxford, pp 79-109. doi: <https://doi.org/10.1016/B978-0-7506-9188-8.50011-5>
- Antonovsky N, Gleizer S, Noor E, Zohar Y, Herz E, Barenholz U, Zelcbuch L, Amram S, Wides A, Tepper N, Davidi D, Bar-On Y, Bareia T, Wernick DG, Shani I, Malitsky S, Jona G, Bar-Even A, Milo R (2016) Sugar synthesis from CO<sub>2</sub> in *Escherichia coli*. Cell 166(1): 115–125. <https://doi.org/10.1016/j.cell.2016.05.064>
- Arfman N, Watling EM, Clement W, van Oosterwijk RJ, de Vries GE, Harder W, Attwood MM, Dijkhuizen L (1989) Methanol metabolism in thermotolerant methylothrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. Arch Microbiol 152(3):280–288
- Balasubramanian R, Smith SM, Rawat S, Yatsunyk LA, Stemmler TL, Rosenzweig AC (2010) Oxidation of methane by a biological dicopper centre. Nature 465(7294):115–119. <https://doi.org/10.1038/nature08992>
- Bar-Even A (2016) Formate assimilation: the metabolic architecture of natural and synthetic pathways. Biochemistry 55(28):3851–3863. <https://doi.org/10.1021/acs.biochem.6b00495>
- Bassham JA, Benson AA, Calvin M (1950) The path of carbon in photosynthesis. J Biol Chem 185(2):781–787
- Becker J, Wittmann C (2015) Advanced biotechnology: metabolically engineered cells for the bio-based production of chemicals and fuels, materials, and health-care products. Angew Chem 54(11):3328–3350. <https://doi.org/10.1002/anie.201409033>
- Bennett RK, Gonzalez JE, Whitaker WB, Antoniewicz MR, Papoutsakis ET (2018) Expression of heterologous non-oxidative pentose phosphate pathway from *Bacillus methanolicus* and phosphoglucose isomerase deletion improves methanol assimilation and metabolite production by a synthetic *Escherichia coli* methylothroph. Metab Eng 45:75–85. <https://doi.org/10.1016/j.ymben.2017.11.016>
- Brautaset T, Heggset M, Marita B, Heux S, Kiefer P, Krog A, Lessmeier L, Muller JE, Portais JC, Potthoff E, Quax WJ, Sibbald M, Vorholt JA, Wendisch VF (2013) Novel methanol dehydrogenase enzymes from *Bacillus*. WO 2013110797, 1 Aug 2013
- Brautaset T, Jakobsen MO, Flickinger MC, Valla S, Ellingsen TE (2004) Plasmid-dependent methylothrophy in thermotolerant *Bacillus methanolicus*. J Bacteriol 186(5):1229–1238
- Burgard AP, Osterhout RE, Van Dien SJ, Tracewell CA, Pharkya P, Andrae S (2014) Microorganisms and methods for enhancing the availability of reducing equivalents in the presence of methanol, and for producing 1,4-butanediol related thereto. US 20140058056 A1, 27 Feb 2014
- Bystrykh LV, Vonck J, van Bruggen EF, van Beeumen J, Samyn B, Govorukhina NI, Arfman N, Duine JA, Dijkhuizen L (1993) Electron microscopic analysis and structural characterization of novel NADP(H)-containing methanol: N,N'-dimethyl-4-nitrosoaniline oxidoreductases from the gram-positive methylothrophic bacteria *Amycolatopsis methanolica* and *Mycobacterium gastri* MB19. J Bacteriol 175(6):1814–1822
- Carbonell P, Currin A, Jervis AJ, Rattray NJ, Swainston N, Yan C, Takano E, Breitling R (2016) Bioinformatics for the synthetic biology of natural products: integrating across the design-build-test cycle. Nat Prod Rep 33(8):925–932. <https://doi.org/10.1039/c6np00018e>
- Chistoserdova L (2011) Modularity of methylothrophy, revisited. Environ Microbiol 13(10): 2603–2622. <https://doi.org/10.1111/j.1462-2920.2011.02464.x>
- Chung BK, Selvarasu S, Andrea C, Ryu J, Lee H, Ahn J, Lee H, Lee DY (2010) Genome-scale metabolic reconstruction and in silico analysis of methylothrophic yeast *Pichia pastoris* for strain improvement. Microb Cell Fact 9:50. <https://doi.org/10.1186/1475-2859-9-50>
- Clomburg JM, Crumbley AM, Gonzalez R (2017) Industrial biomanufacturing: the future of chemical production. Science 355(6320). <https://doi.org/10.1126/science.aag0804>
- Coleman WJ, Vidanes GM, Cottarel G, Muley S, Kamimura R, Javan AF, Sun J, Groban ES (2014) Biological conversion of multi-carbon compounds from methane. US 20140273128 A1, 18 Sep 2014

- Conrado RJ, Gonzalez R (2014) Chemistry. Envisioning the bioconversion of methane to liquid fuels. *Science* 343(6171):621–623. <https://doi.org/10.1126/science.1246929>
- Dai Z, Gu H, Zhang S, Xin F, Zhang W, Dong W, Ma J, Jia H, Jiang M (2017) Metabolic construction strategies for direct methanol utilization in *Saccharomyces cerevisiae*. *Bioresour Technol* 245:1407–1412. <https://doi.org/10.1016/j.biortech.2017.05.100>
- Dedysh SN, Naumoff DG, Vorobev AV, Kyrpides N, Woyke T, Shapiro N, Crombie AT, Murrell JC, Kalyuzhnaya MG, Smirnova AV, Dunfield PF (2015) Draft genome sequence of *Methyloferula stellata* AR4, an obligate methanotroph possessing only a soluble methane monoxygenase. *Genome Announc* 3(2). <https://doi.org/10.1128/genomeA.01555-14>
- Ekeroth L, Villadsen J (1991) Chapter 9 – Single cell protein production from C1 compounds. In: Israel Goldberg, J. Stefan Rokem. *Biology of methylotrophs*. Butterworth-Heinemann: Oxford, pp 205–231. doi:<https://doi.org/10.1016/B978-0-7506-9188-8.50016-4>
- Erb TJ, Berg IA, Brecht V, Muller M, Fuchs G, Alber BE (2007) Synthesis of C5-dicarboxylic acids from C2-units involving crotonyl-CoA carboxylase/reductase: the ethylmalonyl-CoA pathway. *Proc Natl Acad Sci USA* 104(25):10631–10636. <https://doi.org/10.1073/pnas.0702791104>
- Erb TJ, Jones PR, Bar-Even A (2017) Synthetic metabolism: metabolic engineering meets enzyme design. *Curr Opin Chem Biol* 37:56–62. <https://doi.org/10.1016/j.cbpa.2016.12.023>
- Ghosh M, Avezoux A, Anthony C, Harlos K, Blake CC (1994) X-ray structure of PQQ-dependent methanol dehydrogenase. *Exs* 71:251–260
- Gilman A, Laurens LM, Puri AW, Chu F, Pienkos PT, Lidstrom ME (2015) Bioreactor performance parameters for an industrially-promising methanotroph *Methylomicrobium buryatense* 5GB1. *Microb Cell Fact* 14:182. <https://doi.org/10.1186/s12934-015-0372-8>
- Gou Z, Xing XH, Luo M, Jiang H, Han B, Wu H, Wang L, Zhang F (2006) Functional expression of the particulate methane mono-oxygenase gene in recombinant *Rhodococcus erythropolis*. *FEMS Microbiol Lett* 263(2):136–141. <https://doi.org/10.1111/j.1574-6968.2006.00363.x>
- Hadadi N, Hafner J, Shajkofci A, Zisaki A, Hatzimanikatis V (2016) ATLAS of biochemistry: a repository of all possible biochemical reactions for synthetic biology and metabolic engineering studies. *ACS Synth Biol* 5(10):1155–1166. <https://doi.org/10.1021/acssynbio.6b00054>
- Hakemian AS, Rosenzweig AC (2007) The biochemistry of methane oxidation. *Annu Rev Biochem* 76:223–241. <https://doi.org/10.1146/annurev.biochem.76.061505.175355>
- Haynes CA, Gonzalez R (2014) Rethinking biological activation of methane and conversion to liquid fuels. *Nat Chem Biol* 10(5):331–339. <https://doi.org/10.1038/nchembio.1509>
- Helman N, Clarke E, Greenfield D (2015) Synthetic methanotrophic and methylotrophic microorganisms. *WO 2015160848 A1*, 22 Oct 2015
- Hibi Y, Asai K, Arafuka H, Hamajima M, Iwama T, Kawai K (2011) Molecular structure of La3+-induced methanol dehydrogenase-like protein in *Methylobacterium radiotolerans*. *J Biosci Bioeng* 111(5):547–549. <https://doi.org/10.1016/j.jbiosc.2010.12.017>
- Irla M, Heggeset TM, Naerdal I, Paul L, Haugen T, Le SB, Brautaset T, Wendisch VF (2016) Genome-based genetic tool development for *Bacillus methanolicus*: theta- and rolling circle-replicating plasmids for inducible gene expression and application to methanol-based cadaverine production. *Front Microbiol* 7:1481. <https://doi.org/10.3389/fmicb.2016.01481>
- Jeffryes JG, Colastani RL, Elbadawi-Sidhu M, Kind T, Niehaus TD, Broadbelt LJ, Hanson AD, Fiehn O, Tyo KE, Henry CS (2015) MINEs: open access databases of computationally predicted enzyme promiscuity products for untargeted metabolomics. *J Cheminf* 7:44. <https://doi.org/10.1186/s13321-015-0087-1>
- Kalyuzhnaya MG, Puri AW, Lidstrom ME (2015) Metabolic engineering in methanotrophic bacteria. *Metab Eng* 29:142–152. <https://doi.org/10.1016/j.ymben.2015.03.010>
- Kolb S (2009) Aerobic methanol-oxidizing bacteria in soil. *FEMS Microbiol Lett* 300(1):1–10. <https://doi.org/10.1111/j.1574-6968.2009.01681.x>
- Koopman FW, de Winde JH, Ruijsenaars HJ (2009) C(1) compounds as auxiliary substrate for engineered *Pseudomonas putida* S12. *Appl Microbiol Biotechnol* 83(4):705–713. <https://doi.org/10.1007/s00253-009-1922-y>

- Lessmeier L, Pfeifenschneider J, Carnicer M, Heux S, Portais JC, Wendisch VF (2015) Production of carbon-13-labeled cadaverine by engineered *Corynebacterium glutamicum* using carbon-13-labeled methanol as co-substrate. *Appl Microbiol Biotechnol*. <https://doi.org/10.1007/s00253-015-6906-5>
- Lidstrom ME (2006) Aerobic methyloctrophic prokaryotes. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds) *The prokaryotes, Ecophysiology and biochemistry*, vol 2. Springer New York, New York, NY, pp 618–634. [https://doi.org/10.1007/0-387-30742-7\\_20](https://doi.org/10.1007/0-387-30742-7_20)
- Lloyd JS, De Marco P, Dalton H, Murrell JC (1999) Heterologous expression of soluble methane monooxygenase genes in methanotrophs containing only particulate methane monooxygenase. *Arch Microbiol* 171(6):364–370
- Lynch M (2014) Microorganisms for the conversion of methane and methanol to higher value chemicals and fuels. WO 2014165763 A1, 9 Oct 2014
- Matelbs RI, Tannenbaum SE (1968) Single-Cell protein. *Econ Bot* 22(1):42–50. <https://doi.org/10.1007/bf02897743>
- Medema MH, van Raaphorst R, Takano E, Breitling R (2012) Computational tools for the synthetic design of biochemical pathways. *Nat Rev Microbiol* 10(3):191–202. <https://doi.org/10.1038/nrmicro2717>
- Merkx M, Kopp DA, Sazinsky MH, Blazyk JL, Muller J, Lippard SJ (2001) Dioxygen activation and methane hydroxylation by soluble methane monooxygenase: a tale of two irons and three proteins A list of abbreviations can be found in Section 7. *Angew Chem* 40(15):2782–2807
- Mes TZD, de Stams AJM, Zeeman G (2003) Chapter 4. Methane production by anaerobic digestion of wastewater and solid wastes. In: Reith JH, Wijffels RH, Barten H (eds) *Biomethane and biohydrogen: status and perspectives of biological methane and hydrogen production*. Dutch Biological Hydrogen Foundation – NOVEM, The Hague, The Netherlands, pp 58–94
- Methanol Institute (1989) <http://www.methanol.org>. Accessed 20 Jan 2016
- Muller JE, Meyer F, Litsanov B, Kiefer P, Vorholt JA (2015a) Core pathways operating during methyloctrophy of *Bacillus methanolicus* MGA3 and induction of a bacillithiol-dependent detoxification pathway upon formaldehyde stress. *Mol Microbiol* 98(6):1089–1100. <https://doi.org/10.1111/mmi.13200>
- Muller JE, Meyer F, Litsanov B, Kiefer P, Potthoff E, Heux S, Quax WJ, Wendisch VF, Brautaset T, Portais JC, Vorholt JA (2015b) Engineering *Escherichia coli* for methanol conversion. *Metab Eng* 28:190–201. <https://doi.org/10.1016/j.ymben.2014.12.008>
- Murrell JC (2002) Expression of soluble methane monooxygenase genes. *Microbiology* 148(Pt 11): 3329–3330. <https://doi.org/10.1099/00221287-148-11-3329>
- Murrell JC, Gilbert B, McDonald IR (2000) Molecular biology and regulation of methane monooxygenase. *Arch Microbiol* 173(5–6):325–332
- Naerdal I, Pfeifenschneider J, Brautaset T, Wendisch VF (2015) Methanol-based cadaverine production by genetically engineered *Bacillus methanolicus* strains. *Microb Biotechnol* 8(2): 342–350. <https://doi.org/10.1111/1751-7915.12257>
- Nguyen AD, Hwang IY, Chan JY, Lee EY (2016) Reconstruction of methanol and formate metabolic pathway in non-native host for biosynthesis of chemicals and biofuels. *Biotechnol Bioproc* E 21(4):477–482. <https://doi.org/10.1007/s12257-016-0301-7>
- Olah GA (2013) Towards oil independence through renewable methanol chemistry. *Angew Chem Int Ed* 52(1):104–107. <https://doi.org/10.1002/anie.201204995>
- Orita I, Sakamoto N, Kato N, Yurimoto H, Sakai Y (2007) Bifunctional enzyme fusion of 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase. *Appl Microbiol Biotechnol* 76(2):439–445. <https://doi.org/10.1007/s00253-007-1023-8>
- Papoutsakis ET, Nicolaou S, Fast A, Falara V, Bennett RK, Whitaker WB, Sandoval NR, Gonzalez J, Antoniewicz M (2015) Synthetic methyloctrophy to liquid fuels and chemicals. WO 2015/108777 A1, 23 Jul 2015
- Peyraud R, Kiefer P, Christen P, Massou S, Portais JC, Vorholt JA (2009) Demonstration of the ethylmalonyl-CoA pathway by using <sup>13</sup>C metabolomics. *Proc Natl Acad Sci USA* 106(12): 4846–4851. <https://doi.org/10.1073/pnas.0810932106>

- Pfeifenschneider J, Brautaset T, Wendisch VF (2017) Methanol as carbon substrate in the bio-economy: Metabolic engineering of aerobic methylotrophic bacteria for production of value-added chemicals. *Biofuels Bioprod Biorefin* 11(4):719–731. <https://doi.org/10.1002/bbb.1773>
- Planson AG, Carbonell P, Grigoras I, Faulon JL (2012) A retrosynthetic biology approach to therapeutics: from conception to delivery. *Curr Opin Biotechnol* 23(6):948–956. <https://doi.org/10.1016/j.copbio.2012.03.009>
- Pol A, Barends TR, Dieltj A, Khadem AF, Eygensteyn J, Jetten MS, Op den Camp HJ (2014) Rare earth metals are essential for methanotrophic life in volcanic mudpots. *Environ Microbiol* 16(1): 255–264. <https://doi.org/10.1111/1462-2920.12249>
- Price JV, Chen L, Whitaker WB, Papoutsakis E, Chen W (2016) Scaffoldless engineered enzyme assembly for enhanced methanol utilization. *Proc Natl Acad Sci USA*. <https://doi.org/10.1073/pnas.1601797113>
- Quayle JR (1972) The metabolism of one-carbon compounds by micro-organisms. In: Rose AH, Tempest DW (eds) *Advances in microbial physiology*, vol 7. Academic Press, San Diego, CA, pp 119–203. [https://doi.org/10.1016/S0065-2911\(08\)60078-8](https://doi.org/10.1016/S0065-2911(08)60078-8)
- Russmayer H, Buchetics M, Gruber C, Valli M, Grillitsch K, Modarres G, Guerrasio R, Klavins K, Neubauer S, Drexler H, Steiger M, Troyer C, Al Chalabi A, Krebichl G, Sonntag D, Zellnig G, Daum G, Graf AB, Altmann F, Koellensperger G, Hann S, Sauer M, Mattanovich D, Gasser B (2015) Systems-level organization of yeast methylotrophic lifestyle. *BMC Biol* 13(1):80. <https://doi.org/10.1186/s12915-015-0186-5>
- Schrader J, Schilling M, Holtmann D, Sell D, Filho MV, Marx A, Vorholt JA (2009) Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. *Trends Biotechnol* 27(2):107–115. <https://doi.org/10.1016/j.tibtech.2008.10.009>
- Selvakumar G, Nazim S, Kundu S (2008) Chapter 4: Methylotrophy in bacteria. Concept and significance. In: Saikia R (ed) *Microbial biotechnology*. New India Publishing, New Delhi, pp 67–85
- Siegel JB, Smith AL, Poust S, Wargacki AJ, Bar-Even A, Louw C, Shen BW, Eiben CB, Tran HM, Noor E, Gallaher JL, Bale J, Yoshikuni Y, Gelb MH, Keasling JD, Stoddard BL, Lidstrom ME, Baker D (2015) Computational protein design enables a novel one-carbon assimilation pathway. *Proc Natl Acad Sci USA* 112(12):3704–3709. <https://doi.org/10.1073/pnas.1500545112>
- Strong PJ, Xie S, Clarke WP (2015) Methane as a resource: can the methanotrophs add value? *Environ Sci Technol* 49(7):4001–4018. <https://doi.org/10.1021/es504242n>
- Schwander T, Schada von Borzyskowski L, Burgener S, Cortina NS, Erb TJ (2016) A synthetic pathway for the fixation of carbon dioxide in vitro. *Science* 354(6314):900–904. <https://doi.org/10.1126/science.aah5237>
- Tai YS, Zhang K (2015) Enzyme pathways: C1 metabolism redesigned. *Nat Chem Biol* 11(6): 384–386. <https://doi.org/10.1038/nchembio.1819>
- Tani Y (1991) Chapter 11 – Production of useful chemicals by methylotrophs. In: *Biology of methylotrophs*. Butterworth-Heinemann, Oxford, pp 253–270. <https://doi.org/10.1016/B978-0-7506-9188-8.50018-8>
- de la Torre A, Metivier A, Chu F, Laurens LM, Beck DA, Pienkos PT, Lidstrom ME, Kalyuzhnaya MG (2015) Genome-scale metabolic reconstructions and theoretical investigation of methane conversion in *Methylobacterium buryatense* strain 5G(B1). *Microb Cell Fact* 14:188. <https://doi.org/10.1186/s12934-015-0377-3>
- Vieira G, Carnicer M, Portais JC, Heux S (2014) FindPath: a Matlab solution for in silico design of synthetic metabolic pathways. *Bioinformatics* 30(20):2986–2988. <https://doi.org/10.1093/bioinformatics/btu422>
- Vorholt JA (2002) Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. *Arch Microbiol* 178(4):239–249. <https://doi.org/10.1007/s00203-002-0450-2>
- Wendisch VF, Brito LF, Gil Lopez M, Hennig G, Pfeifenschneider J, Sgobba E, Veldmann KH (2016) The flexible feedstock concept in industrial biotechnology: metabolic engineering of *Escherichia coli*, *Corynebacterium glutamicum*, *Pseudomonas*, *Bacillus* and yeast strains for

- access to alternative carbon sources. *J Biotechnol* 234:139–157. <https://doi.org/10.1016/j.jbiotec.2016.07.022>
- West CA, Salmond GP, Dalton H, Murrell JC (1992) Functional expression in *Escherichia coli* of proteins B and C from soluble methane monooxygenase of *Methylococcus capsulatus* (Bath). *J Gen Microbiol* 138(7):1301–1307. <https://doi.org/10.1099/00221287-138-7-1301>
- Whitaker WB, Jones JA, Bennett RK, Gonzalez JE, Vernacchio VR, Collins SM, Palmer MA, Schmidt S, Antoniewicz MR, Koffas MA, Papoutsakis ET (2017) Engineering the biological conversion of methanol to specialty chemicals in *Escherichia coli*. *Metab Eng* 39:49–59. <https://doi.org/10.1016/j.ymben.2016.10.015>
- Whitaker WB, Sandoval NR, Bennett RK, Fast AG, Papoutsakis ET (2015) Synthetic methylophony: engineering the production of biofuels and chemicals based on the biology of aerobic methanol utilization. *Curr Opin Biotechnol* 33:165–175. <https://doi.org/10.1016/j.copbio.2015.01.007>
- Witthoff S, Schmitz K, Niedenfuhr S, Noh K, Noack S, Bott M, Marienhagen J (2015) Metabolic engineering of *Corynebacterium glutamicum* for methanol metabolism. *Appl Environ Microbiol* 81(6):2215–2225. <https://doi.org/10.1128/AEM.03110-14>
- Wood TK (2002) Active expression of soluble methane monooxygenase from *Methylosinus trichosporium* OB3b in heterologous hosts. *Microbiology* 148(Pt 11):3328–3329. <https://doi.org/10.1099/00221287-148-11-3328>
- Yurimoto H, Kato N, Sakai Y (2009) Genomic organization and biochemistry of the ribulose monophosphate pathway and its application in biotechnology. *Appl Microbiol Biotechnol* 84(3):407–416. <https://doi.org/10.1007/s00253-009-2120-7>
- Yurimoto H, Oku M, Sakai Y (2011) Yeast methylophony: metabolism, gene regulation and peroxisome homeostasis. *Int J Microbiol* 2011:101298. <https://doi.org/10.1155/2011/101298>