

# Microbial Methylotrophic Metabolism: Recent Metabolic Modeling Efforts and Their Applications In Industrial Biotechnology

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# Microbial Methylotrophic Metabolism: Recent Metabolic Modeling Efforts and Their Applications In Industrial Biotechnology

*Christian Lieven, Markus J. Herrgård, and Nikolaus Sonnenschein**

**Developing methylotrophic bacteria into cell factories that meet the chemical demand of the future could be both economical and environmentally friendly. Methane is not only an abundant, low-cost resource but also a potent greenhouse gas, the capture of which could help to reduce greenhouse gas emissions. Rational strain design workflows rely on the availability of carefully combined knowledge often in the form of genome-scale metabolic models to construct high-producer organisms. In this review, the authors present the most recent genome-scale metabolic models in aerobic methylotrophy and their applications. Further, the authors present models for the study of anaerobic methanotrophy through reverse methanogenesis and suggest organisms that may be of interest for expanding one-carbon industrial biotechnology. Metabolic models of methylotrophs are scarce, yet they are important first steps toward rational strain-design in these organisms.**

## 1. Introduction

Methane, the primary component of shale gas, natural gas and biogas, is an abundant, albeit highly distributed, and small-scale resource.[1] A powerful greenhouse gas, its release from decomposing landfill and agricultural waste, gas flares, and wastewater treatment plants into the atmosphere contributes strongly to global warming.[2] The output from these sites can be captured and the carbon, that is currently wasted, can sustainably be converted into value-added chemicals, fuels or electricity by means of microbial activity. To illustrate, the amount of carbon released from global venting and flaring in 2014 alone would have been sufficient to cover the world's requirement for methanol, ethylene, propylene, butadiene, xylene, benzene, and toluene.[1] In addition to the environmental benefits of carbon capture at these sites, the price of methane is lower and its per-carbon yield higher than that of glucose, making it the ideal substrate for cell factories.[3] Consequently, public and private funding, and thus general research in this area has increased.

Pieja et al.[4] have reviewed a number of studies that explore potential gas-to-products technologies using methanotrophic organisms. Methanol, which is the first product of aerobic methane oxidation, presents a similarly suitable feedstock for biotechnological applications. Strategies involving native methylotrophs have been reviewed by Clomburg et al.,[1] while achievements in synthetic implementations of methylotrophy have been expanded upon by Bennett et al.[2] To rationally improve strain designs of native and synthetic methanotrophs, in silico systems biology tools can be employed.[5] The fundament of many in silico approaches is a formalized representation of an organism's metabolic network in the form of a genome-scale metabolic model (GEM).

Here in this review, we focus on organisms for which GEMs are currently available in literature that could support the development and improvement of industrial producer strains, which convert methane or methanol into value-added compounds. Elsewhere, a similar effort has been carried out investigating genome-scale metabolic models of clostridia, which are the relevant biocatalyst of syngas ($CO_2$, CO, $H_2$) fermentations.[6]

## 2. Underlying Principles of Genome-Scale Metabolic Modeling

Constraints-based reconstruction and analysis (COBRA) of metabolic networks has become a widely adopted discipline of systems biology in the past two decades.[7] From the sequenced genome of any given organism, information about the specific enzymatic reactions can be extracted and translated into a set of stoichiometric equations. These equations are then viewed as a closed system, which is mass-, and ideally, charge-balanced. Moreover, it is assumed that the system is at a steady state, meaning that there is no net accumulation of intracellular material. Based on these premises, the internal metabolic fluxes of an organism can be expressed as: $\mathbf{Sv} = \mathbf{0}$ a linear system of equations where the matrix $\mathbf{S}$ represents all stoichiometric coefficients from the set of enzymatic reactions, and

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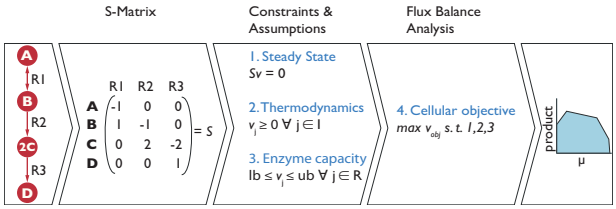


**Figure 1.** Stoichiometric models rely on biochemical and physical assumptions. The stoichiometry of metabolic reactions can be translated into a system of linear equations formally written as a stoichiometric matrix (S-Matrix). Four common assumptions help reduce the solutions that can be found for this system: 1. There is no change in mass over time, 2. The solutions have to be thermodynamically feasible, 3. Enzymes are limited in their capacity, and 4. A cellular objective mimics a specific metabolic phenotype. Together, these assumptions allow i.e., the calculation of product yield at different specific growth rates μ.

the vector **v** represents the flux distribution across all reactions (see **Figure 1**). Flux balance analysis (FBA) can then be used to obtain a specific flux distribution, typically one that maximizes the flux through a specific reaction, for instance the biomass equation.[8]

A well-curated genome-scale metabolic network by itself is a powerful knowledgebase as it accounts for the interconnection between genes, reactions, metabolites, and databases cross-references (meta-information).[9] Built on top of this, FBA and derivative methods have been shown to accurately predict growth phenotypes. Thus, they are useful to prospect strategies for metabolic engineering in silico.[10] One example for the successful integration of a genome-scale metabolic model and metabolic engineering is the development of an *E. coli* strain capable of producing 1,4-butanediol,[11] the design of which relied on the available GEM at the time[12] and an algorithm for the prediction of biological pathways to a specific target compound.[13] The high-level production strain developed based on this design has entered commercial production in 2016 representing one of the rare instances where non-native commodity chemicals are produced by the fermentation route commercially (www.novamont.com/eng/read-press-release/mater-biotech/). Many other fruitful applications of GEMs and COBRA methods have been reported, although chiefly for the model organisms *Escherichia coli* and *Saccharomyces cerevisiae*.[14]

## 3. State of the Art: Existing C1 Metabolic Models

### 3.1. GEMs for Aerobe Methanotrophy

Nature has found two distinct ways of breaking the strong bond between the carbon atom and one of the four hydrogen atoms of methane.[15] In aerobic methanotrophs, two types of methane-monooxygenases can catalyze this reaction, converting methane and oxygen to methanol and water (**Figure 2**). Few organisms express a soluble monooxygenase (sMMO), which receives the electrons necessary for oxidation from NADH. In a majority of methanotrophs, however, the reaction is carried out by a membrane-bound, so called particulate methane-monooxygenase (pMMO). While it also requires two electrons to carry out the oxidation of methane, its native reductant is still debated.[16] Three possible scenarios regarding the mode of electron transfer

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**Nikolaus Sonnenschein** is an in silico strain engineer and group leader at The Novo Nordisk Foundation Center for Biosustainability at the Technical University of Denmark. His group develops software for computer-aided cell factory design that integrates omics data with mathematical models of host organisms. His group is furthermore working on fully automating cell factory development by moving experimentation to the cloud. Before moving to Denmark, he worked as a postdoc in the Systems Biology Research Group at University of California, San Diego and received a PhD in Bioinformatics from Jacobs University Bremen in Germany.

 

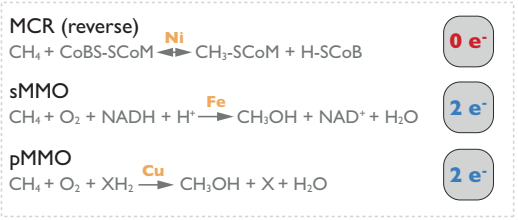


**Figure 2.** Comparison of the stoichiometry of MCR, sMMO, and pMMO. Nickel (Ni), Iron (Fe) and Copper (Cu) activate a C-H bond of methane in MCR, sMMO and pMMO respectively. While the sMMO and pMMO require two electrons to activate molecular oxygen, the anaerobic methane oxidation catalyzed by MCR does not require additional energy. This figure was adapted from [35]gr2

can be considered (**Figure 3**): 1) electrons needed for the oxidation of methane are supplied by NADH produced from formaldehyde oxidation further downstream, while the electrons from methanol oxidation are shuttled into ATP production via a *redox arm* composed of cytochromes; 2) The pMMO is directly coupled to the methanol dehydrogenase, which allows an immediate exchange of electrons between the two reactions; 3) In the so called uphill electron transfer electrons are supplied to the pMMO by MDH and NADH.[17]

With the genome-scale metabolic model of *Methylomicrobium buryatense* 5G(B1), de la Torre et al.[18] presented the first ever manually curated GEM of a methane-utilizing bacterium. Using the model to explore each mode of electron transfer, they were able to eliminate the *redox arm* hypothesis as it correlated the least with their experimental measurements for *M. buryatense*. Further, the researchers found that the in silico replacement of the pyruvate dehydrogenase with a phosphoketolase did not improve the overall carbon yield on methane. Instead a decrease in carbon yield was predicted since more methane was oxidized to $CO_2$ as a source of NADH. The genome scale metabolic model complements the genetic tools that have already been established for *M. buryatense*,[19] and recent successes in metabolic engineering show that there is potential to commercialize the production of chemicals in *M. buryatense*. For instance, Dong et al.[20] have been able to increase the production of membrane phospholipids, which they subsequently

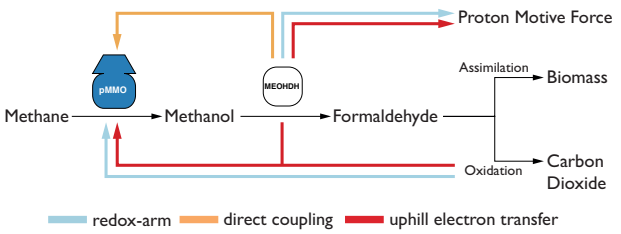


**Figure 3.** Three hypotheses describe the electron transfer to the particulate Methane Monooxygenase. 1. Redox-arm: Electrons released from the methanol dehydrogenase (MEOHDH) contribute to building up the protein motive force. The oxidation of formaldehyde to carbon dioxide provides the electrons for the oxidation of methane. 2. Direct coupling: The MEOHDH passes electrons directly to the pMMO. 3. Uphill electron transfer: Electrons from the MEOHDH can reach both the terminal oxidase and the pMMO.

processed into diesel blendstock, and Henard et al.[21] have used *M. buryatense* to produce lactate at a yield of 0.05 g lactate/g methane. In addition, the latter have been able to improve the lipid and biomass yield 2.6-fold by overexpressing the phosphoketolase.[22]

A GEM of the halotolerant *Methylomicrobium alcaliphilum* 20ZR is also available from the Kalyuzhnaya Lab.[23] The authors gathered metabolomics profiles of *M. alcaliphilum* grown on methane and methanol to verify and improve the in silico predictions. The model correctly predicted an increase in the metabolite pools of amino acids when grown on methanol instead of methane. Furthermore, simulations indicated that during oxygen-limited growth pyrophosphate-dependent reactions play an important role to improve the biomass yield. Lastly, they determined that a direct coupling electron transfer mode fit best their observations. In previous studies, the organism was found to ferment methane-derived formaldehyde to organic acids at low oxygen tension,[24] and was used to investigate the biosynthesis and degradation pathways of sucrose in methanotrophs.[25] Hill et al.[26] successfully co-cultivated *M. alcaliphilum* 20Z with *Synechococcus* PCC 7002 on a mixture of methane and carbon dioxide. This approach avoids the need to control the ratio of methane to oxygen like it is the case in methanotroph monocultures. A promising industrial application involving *M. alcaliphilum* 20Z is the production of the osmolyte and biostabilizer ectoine. One kilogram of ectoine costs around US$ 1000, with the average global demand of the pharmaceutical industry amounting to 15 000 tonnes per year.[27] The GEM for *M. alcaliphilum* 20ZR could be employed to evaluate ectoine production and develop hypothesis-driven strain engineering strategies in this organism.

The production of animal feed from natural gas using *Methylococcus capsulatus* as the provider of single cell protein (SCP) had already been commercialized in the 70s until a drop in oil prices made these efforts economically infeasible. Today, at least two companies produce SCP in this manner at pilot scale with commercial scale plants in the process of being constructed, among them US-based Calysta (www.calysta.com) and Denmark-based Unibio (www.unibio.dk).[28] In addition to SCP, early stage work has been done to develop *M. capsulatus* as a production host for several commodity chemicals. Examples include propylene by Calysta,[29] succinate by String Bio,[30] 1,4-butanediol by Sekisui Chemical[31] and other multi-carbon compounds by the Intrexon Corporation.[32]

Although *M. capsulatus* has been extensively studied in the past 50 years,[33] a curated GEM was only recently completed by a group that included the authors of the present review.[34] We computationally predicted transporter genes and assigned them to corresponding transport reactions. Similar to the efforts carried out by de la Torre et al.[18] for *M. buryatense*, we investigated which mode of electron transfer best represents measured parameters for *Methylococcus capsulatus*. We found that simulations of the three modes exclusively could not adequately represent the experimentally observed ratio of $O_2$ uptake per mol of methane. Only by reducing the efficiency of the uphill electron transfer mode we were able to replicate the reference ratio. Moreover, we found that the energetic burden of $NH_4$ oxidation to $NO_2$ by the pMMO likely affects this ratio, when cells are grown on medium containing $NH_4$ as the nitrogen source. To facilitate visual inspection of multi-omics data and more intuitive exploration of the metabolic potential, we

 

also provide a metabolic map that displays the metabolic network described by the model.

### 3.2. GEMs for Methylotrophy

Despite being more reduced, the hypothetical per carbon substrate yields of methane have been determined to be consistently lower than those of methanol in an in silico study carried out by Comer et al.[3] This is due to the low efficiency conversion catalyzed by the methane monooxygenase. Natural aerobic methane oxidation requires two electrons, which subsequently have to be recovered by the methanol dehydrogenase, oxidizing methanol to formaldehyde, or further downstream depending on the mode of electron transfer.[1] This contributes to losing 36% of the energy within the highly reduced molecule by an essentially redox-neutral conversion of methane to formaldehyde.[35] Methanol is an intermediate of methanotrophy, therefore many methanotrophs can use it as their sole carbon and energy source. Since it is a liquid, using methanol bypasses potential issues with mass-transfer during gas-fermentation. Although less environmentally friendly than biocatalysis, a recent breakthrough allows the production of methanol by way of chemical conversion from methane at milder conditions than previously possible.[36] This could increase the role of methanol as a one-carbon feedstock.

The metabolism of the facultative methylotroph *Methylobacterium extorquens* AM1 has been studied in detail since well over 50 years.[37] The considerable research interest has resulted in the development of genetic tools and protocols, ultimately leading to the establishment of several production processes, the products of which include polyhydroxyalkanoates (PHA), serine, dicarboxylic acids derived from the ethylmalonyl-CoA pathway, alcohols, and proteins.[38] A GEM was established for *M. extorquens* AM1 to investigate the topology and operation of the intertwined metabolic cycles that operate in the bacterium when grown on methanol.[39] In a separate study, researchers succeeded in heterologously producing 1.65 g $L^{-1}$ of α-homulene, an anti-inflammatory terpenoid, in *M. extorquens*. The metabolic model was used to calculate the maximum theoretical yield of the compound.

### 3.3. GEMs for Anaerobic Methanotrophy

In addition to the energy loss caused by the oxygen-dependent conversion of methane mentioned above, the volumetric mass transfer of methane and oxygen is another limitation especially at large-scale operation. Although innovative specialized reactor designs alleviate the issue,[40] they translate into increased capital expenses when compared to using regular stirred-tank vessels. With respect to these drawbacks, anaerobic production of chemicals from methane is considered more ideal, despite the issue with lower growth rates and productivities of microbes under these conditions.[2]

The methyl-coenzyme M reductase (MCR) is the key enzyme required for the anaerobic biosynthesis of methane. In this reaction, methyl-coenzyme M (methyl-SCoM) reacts with coenzyme B (CoBSH) to form methane and COBS-SCoM.[16] Anaerobic methanotrophs (ANME) have a homolog MCR that is able to catalyze the reverse reaction (Figure 2). In nature, ANME grow in consortia with syntrophic bacteria that participate in the removal of reducing equivalents, which has complicated the isolation and culturing of native ANME strains.[2] However, through metagenomic sequencing it was recently possible to obtain the corresponding MCR gene, successfully clone, and express it in the methane-producing archaeon *Methanosarcina acetivorans* C2A.[41] Nazem-Bokaee et al. published iMAC868,[42] an update to the two previously existing GEMs for *M. acetivorans,* iVS941[43] and iMB754.[44] Using iMAC868, the authors studied the feasibility of producing acetate, formate and pyruvate on methane as a function of $Fe^{3+}$ reduction. They further improved the predictions of growth yield on the native substrates methanol and acetate, in addition to making the necessary changes to enable methanotrophy. The authors predicted the hypothetically maximal biomass yields and the yields of biofuel precursors methanol, ethanol, butanol, and isobutanol on methane. Considering the ΔG of different external electron acceptors, they found that the yields were highest for $Fe^{3+}$ reduction when $CO_2$ in the form of bicarbonate was co-utilized. The native products of reverse methanogenesis in *A. acetivorans* were determined to be acetate and $CO_2$. Using the same engineered host, McAnulty et al.[45] produced lactate yielding 0.59 g per gram of methane. This is an order of magnitude greater than the previously reported yield of lactate on methane in an aerobic process.[21]

Bennett et al.[2] suggest that as there are genetic tools available for it, the hydrogenotrophic methanogen *Methanococcus maripaludis* could also be consider as a host for reverse methanogenesis. Several metabolic models currently have been reconstructed for this archaeon with the most recent one being iMR539.[46]

### 3.4. Approaches for Synthetic Methanotrophy

Slow growth rates, inefficient molecular techniques and a lack of experience compared to model microorganisms complicate the work with native aerobic and anaerobic methanotrophs. While the implementation of aerobic methane oxidation using pMMO or sMMO has been difficult,[47,48] the transfer of precursor pathways belonging to anaerobic methanotrophy has been successful,[49] thus making the prospect of synthetic anaerobic methanotrophy promising. Most progress, however, has been made with the heterologous expression of methylotrophic genes in the microbial workhorses *Escherichia coli, Corynebacterium glutamicum* and *Saccharomyces cerevisiae*.[2] It is no surprise that GEMs for these three well-established model organisms exist and are continuously updated. For reference, the most recent versions are listed in **Table 1**.

Another promising option, which can be regarded as a step toward heterologous methanotrophy, is the development of synthetic pathways involving novel enzymes.[10] The formolase pathway, which has been constructed around the computationally designed enzyme formolase (FLS), is such a pathway. Overall, the five-step, linear pathway catalyzes the carboligation of three formate molecules into the common three-carbon intermediate dihydroxyacetylphosphate (DHAP) and has been

 

**Table 1.** Genome-scale metabolic models relevant to methano- or methylotrophy.

| Organism | Model ID | Previous Versions | # Reactions | # Metabolites | # Genes | Reference |
|---|---|---|---|---|---|---|
| Aerobic | | | | | | |
| *Methylobacterium extorquens AM1* | iRP911[a)] | First | 1139 | 977 | 911 | [63] |
| *Methylococcus capsulatus Bath* | iMcBath [a, c)] | First | 898 | 877 | 730 | [34] |
| *Methylomicrobium alcaliphilum 20Z* | iIA407 [b)] | First | 433 | 423 | 407 | [23] |
| *Methylomicrobium buryatense 5G(B1)* | iMb5G(B1)[a, b)] | First | 402 | 403 | 314 | [18] |
| Anaerobic (Chassis for Reverse Methanogenesis) | | | | | | |
| *Methanococcus maripaludis* S2 | iMM518[b)] | First | 570 | 556 | 518 | [56] |
| *Methanococcus maripaludis* S2 | iMR539[a, c)] | Independent from iMM518 | 688 | 710 | 539 | [46] |
| *Methanosarcina acetivorans* C2A | iMAC868[b)] | iVS941[d)], iMB745[e)] | 845 | 718 | 868 | [42] |
| Sythetic Methano- or Methylotrophy | | | | | | |
| *Corynebacterium glutamicum* | iCW773[b)] | Reviewed in [64] | 1207 | 950 | 773 | [65] |
| *Eschericia coli* | iML1515[a)] | Reviewed in [66] | 2712 | 1877 | 1516 | [67], Used in [3,68] |
| *Sacharromyces cerevisiae* | YEAST 7[a, c)] | Reviewed in [69] | 3493 | 2220 | 909 | [70], Used in [3] |

[a)] Available as SBML; [b)]Available as XLS; [c)]Available as MAT, [d)][43]; [e)][44].

shown to function in vitro.[50] The authors used flux balance analysis and the core metabolic model of *E. coli*[51] to compare the performance of their novel pathway relative to all natural formate assimilation pathways. They found that the hypothetical maximum biomass yield of the formolase pathway is the second highest (6.5 g cell dry weight/mol formate) behind the reductive TCA cycle (6.7 g cell dry weight/mol formate), but exceeds all other pathways when considering the chemical driving force.

## 4. Conclusions

Although the true impact of genome-scale metabolic models in a specific field of research is hard to quantify, their general success is indisputable.[52] A well-curated GEM is particularly useful for exploring the topology and systems properties of metabolism. In addition, a GEM establishes a connection between stoichiometric, genetic, and meta-information which is the foundation of many strain-design methods. However, there are systemic limitations. A high-quality GEM requires the existence of a carefully annotated genome sequence. An incomplete sequence or erroneous annotation will introduce a bias in the resulting model.[53] The steady state assumption means that dynamic processes such as a change in metabolite concentration cannot be accounted for. Similarly, the effects of regulation can only be applied deliberately e.g., through the use of additional constraints.[34]

While four available GEMs for aerobic methyl- and methanotrophy reported here have been used to explore metabolic interconnections and the system's behavior in specific conditions using constraint-based modeling, further cross validation against experimental data could improve their predictiveness. As

**Table 2.** Methanotrophs that are potentially relevant as biotechnological producers.

| Organism | Genome Sequence | Primary Interest |
|---|---|---|
| *Methylobacter marinus* 7C | [71] | Identification of the ectoine biosynthesis genes[72] |
| *Methylobacterium organophilum* CZ-2 | Not sequenced | Production of PHB[73] and triacylglycerides[27] |
| *Methylocaldum sp. SAD2* | Not sequenced | Production of methanol on high levels of $H_2S$[74] |
| *Methylocapsa acidiphila* | Direct submission NZ_ATYA00000000.1 | Potential production of PHB[75] |
| *Methylocella tundrae* | Not sequenced | Production of methanol[76] |
| *Methylocystis bryophila* | Direct submission NZ_CP019948.1 | Production of methanol[77] |
| *Methylocystis parvus OBBP* | [78] | Production of PHB[79] |
| *Methylocystis sp.* WRRC1 | Not sequenced | Production of a copolymer of PHB and hydroxyvalerate[80] |
| *Methylomicrobium kenyense* AMO1 | Not sequenced | Identification of the ectoine biosynthesis genes[72] |
| *Methylomonas denitrificans* | [81] | Production of $N_2O$ coupled to methane oxidation under hypoxia [81]. |
| *Methylomonas sp.* 16a | Sequenced by DuPont, unpublished | Synthesis of C30 carotenoids,[82] production of astaxanthin and canthaxanthin[83,84] |
| *Methylosinus sporium* | Not sequenced | Production of methanol[85] |
| *Methylosinus trichosporium* OB3b | [86] | Production of methanol[87] |

PHB, polyhydroxybutyrate.

 

such the analysis of model predictions versus data from genetic perturbation (i.e., knockout) experiments in addition to growth studies is an invaluable step.[9,54]

Promiscuous enzyme functions are often not included in biochemical databases and rarely covered in the genome annotation.[55] While the methanogen models iMM518,[56] iMR539,[46] and iMAC868[42] have been validated using small-scale knockout data, the use of this method of validation for a GEM of a methanotroph has been limited to a single reaction knockout in iIA407.[23] Yet, Richards et al.[46] point out that this is made difficult by a low abundance of suitable gene knockout data.

The application of computational strain design methods in this field has not been reported so far. A host of strain design methods have been thoroughly reviewed by Ng et al.[5] Applying a pathway prediction method such as GEM-Path, for instance, could decrease the time required to create a suitable design for the production of commodity chemicals from methane. The algorithm provided 1271 growth-coupled designs for the production of 20 commodity chemicals in *E. coli*.[57] Using the metabolic models that are currently available for methanotrophs, the production potential of the organisms could already be explored.

Lastly, many of the discussed GEMs are distributed in a non-standard, tabular file format (Table 1). While the MATLAB version of the COBRA Toolbox is able to import and simulate models that come in this format, other software tools rely on the communication of models in the Systems Biology Markup Language (SBML). In fact, Ravikrishnan & Raman (2015) advocate the distribution of models in this de facto community standard, because the use of other formats may decrease reproducibility and the ability to use a GEM with the largest portion of available tools.[58]

As evident from this review, metabolic models of methanotrophy are scarce and their potential yet untapped. In the future, many more organisms than the ones reviewed here could become relevant as methanotrophic producer strains, and thus could benefit from having a GEM available. Potential organisms of interest for methanotrophic production are collected in **Table 2** with references to the corresponding genome sequences and publication describing a potential use of the organism for production.

Although the applicability of the organisms in Table 2 is not certain, merely having access to curated biochemical information could serve the scientific community as a whole. As Monk et al. remark, the coverage of metabolic reactomes has stagnated, since little effort is spent on comprehensively uncovering the metabolic space of an organism, especially with regards to the secondary metabolism.[59] A thorough analysis of these organisms may lead to interesting discoveries similar to, for instance, that of hopanoid production in *M. capsulatus* and *Alicyclobacillus acidocaldarius*.[60]

Moreover, the use of novel formalisms could allow future refinement of the GEMs presented herein. For instance, including reactions that describe the mechanistic processes involved in gene expression and protein biosynthesis allows simulating metabolic tradeoffs in energy allocation and the prediction of the organism's maximum growth rate.[61] Due its complexity the lipid metabolism in GEMs is typically represented by lumped reactions, which involve either an artificial average or the most dominant type of fatty acid. Adding special pseudo-reactions based on data from lipid profiling and fatty acid methyl ester analysis could further help to improve the representation of the lipid metabolism.[62]

Despite the minor shortcomings noted above, the availability of these models can only serve to accelerate the process of discovery. GEMs allow researchers to probe certain properties in silico isolated from the potential challenges associated with slow growth, difficulties to culture an organism, and inefficiencies of molecular techniques or even the lack thereof. Hence, they represent an ideal tool to explore and expand the metabolic potential of methanotrophs.

The first steps, presented here, may lead towards a solid foundation for rational strain-design of methanotrophs, and may help to elucidate their many properties.

## Abbreviations

ANME, anaerobic methanotrophs; CO, carbon monoxide; COBRA, constrained-based reconstruction and analysis; CoBSH, coenzyme B; DHAP, dihydroxyacetylphosphate; FBA, flux balance analysis; FLS, formolase; GEM, genome-scale metabolic model; MCR, methyl-coenzyme M reductase; MDH, methanol dehydrogenase; methyl-SCoM, methyl-coenzyme M; PHA, polyhydroxyalkanoates; PHB, polyhydroxybutyrate; pMMO, particulate Methane Monooxygenase; SCP, single-cell protein; SBML, Systems Biology Markup Language; sMMO, soluble Methane Monooxygenase.

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## Conflict of Interest

Unibio is a collaborator in the "Environmentally Friendly Protein Production (EFPro2)" project. The authors are not funded by consulting said company, and thus declare no financial or commercial conflict of interest.

## Keywords

cell factories, COBRA, metabolic modeling, methylotrophy, one-carbon

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