Systems Biology and Metabolic Modeling of C_1-Metabolism

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7.1 Introduction

Recent developments in experimental technologies have transformed traditional microbial physiology into a data-rich or *-omics* discipline (Kalyuzhnaya et al. 2015; Khadem et al. 2011; Wertz et al. 2012). As a result, it has caused a renaissance of the mathematical analysis of biological systems (Karr et al. 2012) and stimulated the development of systems biology workflows which aim to provide a holistic vision of all cellular functions through genomics, transcriptomics, proteomics, metabolomics, and fluxomic data (Cavill et al. 2015; Covert et al. 2001; Crowther et al. 2008; Haque et al. 2015; Leak and Dalton 1986b; Lee et al. 2006b; Machado and Herrgård 2014; Yizhak et al. 2010). In silico modeling of metabolic systems has become a powerful tool, providing insight into the complex processes in cellular metabolism and their underlying regulatory mechanisms, as well as potentially improving the biotechnological design of microbial strains with desired properties (Alon 2006; Lee et al. 2006b). In this chapter, we provide an overview of the systems biology of methane utilization, as an example of one unique microbial function that has been dissected using -omics technologies. We discuss the most recent advances in large-scale investigation and computational representation of related metabolic networks as well as highlight some challenges for further developments in the field.

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M. G. Kalyuzhnaya, X.-H. Xing (eds.), *Methane Biocatalysis: Paving the Way to Sustainability*, https://doi.org/10.1007/978-3-319-74866-5_7

7.2 Bacterial Methane Metabolism

The ability to use methane, i.e., methanotrophy, has been always considered as one of the most unique microbial functions. For years methanotrophy has been attributed to bacteria, known as methanotrophs, and described for *Alphaproteobacteria*, *Gammaproteobacteria*, and *Verrucomicrobia* (reviewed in Kalyuzhnaya et al. 2015; Trotsenko and Murrell 2008). While the ability of methane oxidation has also been demonstrated for some members of Archaea, in this chapter, we will only cover systems approaches applied to bacterial metabolic networks. The core elements of methane utilization were well established by early 1980s (Anthony 1982). Over the past decade, systems biology approaches helped to refine these established metabolic networks (summarized in Table 7.1). The massive amount of *-omics* information also

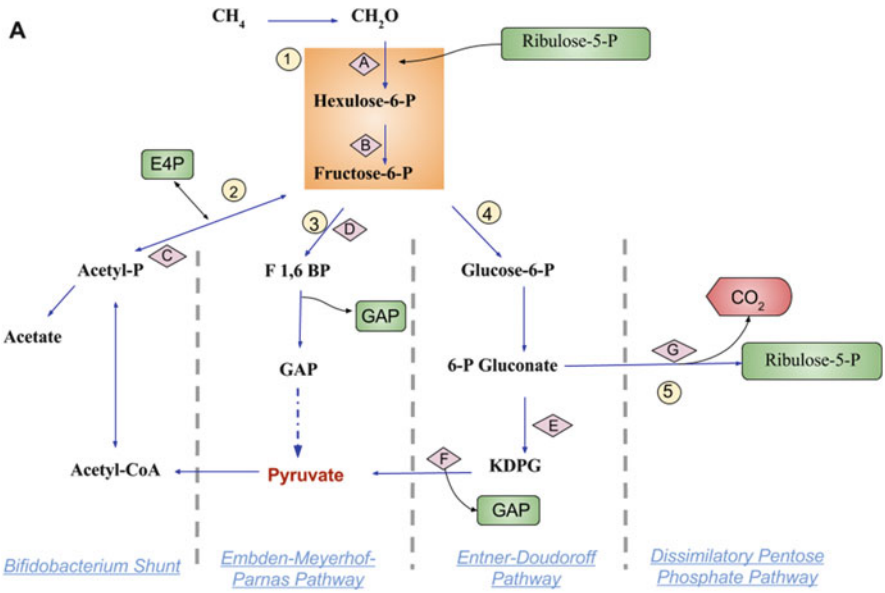
Table 7.1 Overview of system-level investigation of methanotrophy

Omics approach	Species	References
Transciptomics	*Candidatus* Methylomirabilis oxyfera *Methylosinus trichosporium* OB3b *Methylomicrobium alcaliphilum* 20Z *Methylomicrobium buryatense* 5G(B1) *Methylocystis* sp. SB2 *Methylococcus capsulatus* (Bath) *Methyloprofundus sedimenti*	Luesken et al. (2012) Matsen et al. (2013) But et al. (2015), Kalyuzhnaya et al. (2013) Torre et al. (2015) Vorobev et al. (2011) Larsen and Karlsen (2016) Tavormina et al. (2017)
Proteomics	*Methylococcus capsulatus* (Bath) *Methylocella silvestris*	Berven et al. (2006), Kao et al. (2004) Crombie and Murrell (2014)
Metabolomics	*Methylomicrobium alcaliphilum* 20Z *Methylosinus trichosporium* OB3b	Kalyuzhnaya et al. (2013) Yang et al. (2013)
^{13}C flux analysis	*Methylomicrobium buryatense* 5GB1 *Methylomicrobium alcaliphilum* 20Z *Methylosinus trichosporium* OB3b	Fu et al. (2017) Kalyuzhnaya et al. (2013) Yang et al. (2013)
Kinetic models	*Methylosinus trichosporium* OB3b *Methylococcus capsulatus* (Bath) *Methylomicrobium alcaliphilum* 20Z	Yoon and Semrau (2008), Lee et al. (2006) Leak and Dalton (1986b) Akberdin et al. (n.d.)
Genome-scale models	*Methylomicrobium buryatense* 5G(B1) *Methylomicrobium alcaliphilum* 20Z *Methylococcus capsulatus* (Bath)	Torre et al. (2015) Akberdin et al. (2018) Lieven et al. (in preparation)

highlighted a myriad of complexities and exceptions, which continue to challenge our knowledge of methane utilization.

Methanotrophs oxidize methane through the use of copper-dependent particulate methane monooxygenase (pMMO) or iron-linked soluble methane monooxygenase (sMMO) enzymes (Hakemian and Rosenzweig 2007; Culpepper and Rosenzweig 2012; Sirajuddin and Rosenzweig 2015; Chan and Yu 2008). Both enzymes require oxygen and convert methane into methanol, which is further oxidized into formaldehyde by a PQQ-dependent methanol dehydrogenase (MDH) (Chistoserdova 2011). While MDH and its corresponding genes *mxaFI* are the most well-known methanol oxidizers, there are homologues (*xoxF* and *mdh2*) that exist in other species (Kalyuzhnaya et al. 2008; Semrau et al. 2018; reviewed in Chap. 4). Many steps of methanotrophy are interconnected with carbon assimilation (reviewed in Chistoserdova and Lidstrom 2013; Kalyuzhnaya et al. 2015; Trotsenko and Murrell 2008). In some species, formaldehyde is incorporated into fructose 6-phosphate in a two-step reaction driven by one fused or two individual enzymes, hexulose phosphate synthase and isomerase (Orita et al. 2005, 2006; Rozova et al. 2017). These two steps of assimilation are the core of the ribulose monophosphate (RuMP) pathway, which additionally includes pentose phosphate pathway (PPP) reactions and at least one of the glycolytic pathways (Embden–Meyerhof–Parnas, Entner–Doudoroff, or *Bifidobacterium* shunt). Thus, methane-derived carbon enters the canonical sugar catabolic pathways for redox power regeneration or the production of the main precursors for biosynthesis (Fig. 7.1). So far, the RuMP pathway has been found only in gammaproteobacterial methanotrophs. In the majority of known methane-consuming *Alphaproteobacteria*, formaldehyde is first oxidized to formate, which is then incorporated into biomass via tetrahydrofolate-linked $\mathbf{C_1}$-transfer reactions and the serine cycle (Matsen et al. 2013; Yang et al. 2013). It has been postulated that to be an efficient and self-sustained pathway for $\mathbf{C_1}$-carbon utilization, the serine cycle must be coupled with a glyoxylate regeneration pathway, such as the glyoxylate shunt (GS) or ethylmalonyl-coA (EMC) pathway (Anthony 1982; Erb et al. 2007; Fig. 7.2). Both variants of the serine cycle, linked to the GS or EMC pathways, have been identified in methanotrophs. The genetic signatures of the key serine pathway enzymes have also been detected in the genomes of methanotrophic *Gammaproteobacteria* (reviewed in Kalyuzhnaya et al. 2015); however, none of them indicates the presence of a known glyoxylate regeneration pathway. The serine cycle seems to be a functional pathway in bacteria and contributes to carbon assimilation, most likely as a supplementary metabolic module interlinked with the pyruvate node (Kalyuzhnaya et al. 2015; Ward et al. 2004). All methanotrophic *Verrucomicrobia* and some *Proteobacteria* are autotrophs, which assimilate CO_2 via the Calvin cycle (Khadem et al. 2011; Taylor et al. 1981; Vorobev et al. 2011).

All three groups of methanotrophs have functional TCA cycles and quite complex and often redundant sets of electron transfer systems (ETS) (Fig. 7.3). It is predicted that electrons from methane oxidation are transferred to oxygen, regenerating energy for biosynthesis. However, anaerobic respiratory pathways and fermentation pathways have also been described (Kalyuzhnaya et al. 2013; Kits et al. 2015).



B

EMP: 9 CH_2O + 3 NAD^+ + 2 ADP + 5 P_i → 3 PGA + 3 NADH + 3 H^+ + 2 ATP

EDD: 9 CH_2O + 3 NAD^+ + 6 ATP → 3 PGA + 3 NADH + 3 H^+ + 6 ADP + 3 P_i

Bifidobacterium shunt: CH_2O +ADP+ P_i → ATP + Acetate

Dissimilatory Pentose Phosphate Pathway: 6-P Gluconate + NAD^+ → Ru-5-P + CO_2 + NADH

Fig. 7.1 (**a**) The ribulose monophosphate (RuMP) pathway variants. (**b**) Energy and carbon balance of each RuMP. Key steps for aldol condensation of formaldehyde with ribulose monophosphate and isomerization of hexulose 6-phosphate to fructose 6-phosphate. (2–5) Various routes that the fructose 6-phosphate utilizes: (2) the *Bifidobacterium* shunt contributes to fermentation and recycling of acetyl-CoA back to pentose phosphate pathway (PPP), (3) the Embden–Meyerhof–Parnas (EMP) pathway, (4) the Entner–Doudoroff (ED) pathway, (5) the dissimilatory pentose phosphate pathway (dPPP). Green labels indicate intermediates which enter the pentose phosphate pathway (PPP) for regeneration of ribulose 5-phosphate. Key enzymes: (A) hexulose phosphate synthase, (B) hexulose phosphate isomerase, (C) phosphoketolase, (D) PPi-dependent phosphofructokinase, (E) 6-phosphogluconate dehydratase, (F) 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase, (G) 6-phosphogluconate dehydrogenase (decarboxylating)

It has been predicted that the methane oxidation machinery has relatively high basal energy requirements and about 25% of consumed methane is directed toward functions required to sustain a metabolically active state (Akberdin et al. 2018). That might explain the conservation of numerous PPi-dependent reactions in all main

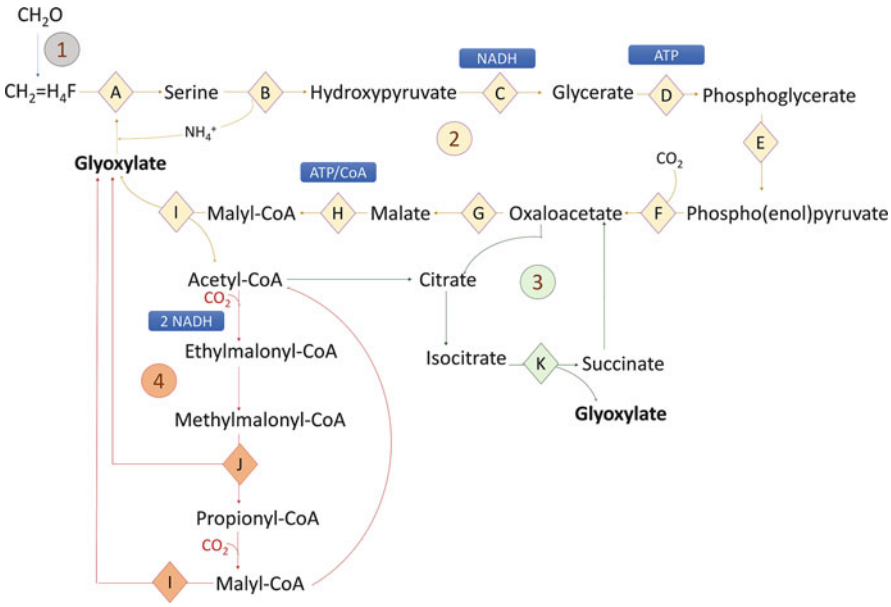


Fig. 7.2 Serine cycle and its variants. (1) Three various pathways have been proposed for methylenetetrahydrofolate formation, including spontaneous condensation with tetrahydrofolate, condensation facilitated by a formaldehyde-activating enzyme (Fae), and tetrahydrofolate-linked C_1-transfer; (2) the core serine pathway involves the assimilation of methylenetetrahydrofolate into phosphoglycerate, which is then used for assimilation and regeneration of C1-accepting molecule, glycine (formed from glyoxylate); (3) the glyoxylate shunt is a truncated TCA cycle allowing replenishment of glyoxylate (Anthony 1982); (4) the ethylmalonyl-CoA cycle is an alternative pathway for glyoxylate regeneration for bacteria lacking isocitrate lyase (Peyraud et al. 2009, 2011; Yang et al. 2013). Key enzymes: (A) serine hydroxymethyltransferase, (B) serine glyoxylate aminotransferase, (C) hydroxypyruvate reductase, (D) glyceratekinase, (E) enolase, (F) phospho (enol)pyruvate (PEP) carboxylase or alternative pathway to converting C_3 to C_4 compounds, (G) malate dehydrogenase, (H) malate thiokinase, (I) malyl-CoA lyase, (J) ß-methylmalyl-CoA lyase, (K) isocitrate lyase

groups of methanotrophs (reviewed in Chap. 6). The estimated growth-dependent energy consumptions fall into a range that is typical for many microbial species (Akberdin et al. 2018).

7.2.1 Genomics

Whole-genome sequencing is a powerful tool that allows one to gain an initial basis for and a holistic insight into the metabolic potential of a studied microorganism. A number of complete or gapped genome sequences for a variety of methanotrophs are now available, which not only provide a fundamental platform for implementation of systems biology strategies for metabolic network reconstruction (Sipkema et al.

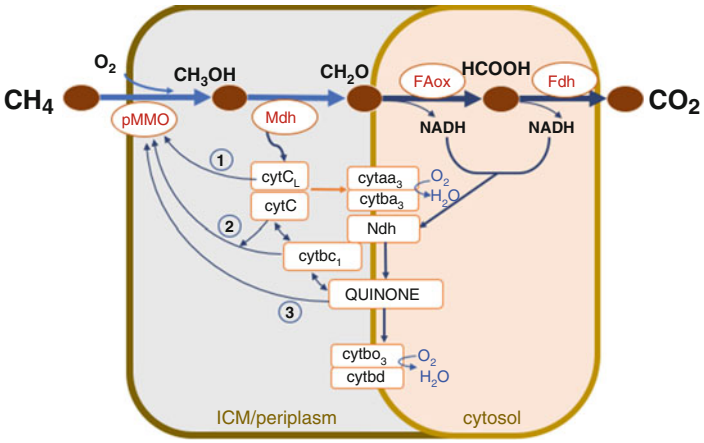


Fig. 7.3 Electron transfer reactions in *Methylomicrobium alcaliphilum* 20Z. 1–3 represent different pathways for redox supply for pMMO reaction: (1) direct coupling mode, (2) uphill electron transfer mode, and (3) redox mode. M. *alcaliphilum* 20Z has various terminal cytochrome oxidases, whose expressions depend on availability of oxygen and copper

2000; Vuilleumier et al. 2009, 2012) but also facilitate comparative and evolutionary analysis of microbial diversity across all genera of methanotrophs (Chistoserdova 2011; Tamas et al. 2014; Tavormina et al. 2017). Genome mining led to the discovery of redundant methanol-utilization systems (Chistoserdova 2011; Chu and Lidstrom 2016; Ettwig et al. 2010), genetic elements for a copper acquisition system (such as methanobactin and its biosynthesis) (Semrau et al. 2013), carbon assimilation pathways in methanotrophic *Verrucomicrobia* (Op den Camp et al. 2009; Anvar et al. 2014; Khadem et al. 2011, 2012), and key elements of nitrogen metabolism, from N_2-fixation to denitrification (Wertz et al. 2012).

The availability of genomes from a variety of pure methanotrophic cultures empowered metagenomics analyses, leading to the identification of molecular and metabolic mechanisms of interactions between methanotrophic strains in complex microbial communities (Beck et al. 2013). Finally, genomic information revealed a number of functions for transfer to and implementation in non-methanotrophic hosts and enabled genome-directed reconstruction and engineering of methanotrophy for biotechnological applications (Fei et al. 2014; Haque et al. 2016; Kalyuzhnaya et al. 2015; Lee and Kim 2015; Henard et al. 2017, also reviewed in Chap. 8).

7.2.2 Transcriptomics

Transcriptomics, or gene-expression profiling, became essential for interpreting genome functionality in a given environmental condition. A number of transcriptomic-based studies have demonstrated how the approach can rapidly advance and facilitate our understanding of the metabolic pathways of C_1-metabolism and its

underlying regulatory mechanisms. In particular, Luesken et al. (2012) used the approach to understand oxygen production and consumption in *Candidatus Methylomirabilis oxyfera*. Transcriptomic data has complemented known enzymatic and genomic information to provide a global overview of the metabolic map for methane assimilation in *Methylosinus trichosporium* OB3b, highlighting the importance of the ethylmalonyl pathways for carbon assimilation (Matsen et al. 2013; Yang et al. 2013). Gene-expression profiles helped to reevaluate C_1-assimilation pathways in *Methylomicrobium alcaliphilum* 20Z (Kalyuzhnaya et al. 2013) and were essential for the discovery of fermentation pathways and the reconstruction of the complete network for sucrose metabolism (But et al. 2015). Detailed transcriptomic analysis of the facultative methanotroph *Methylocystis* sp. strain SB2 (Vorobev et al. 2014) provided insights into pathways for C_2-carbon utilization and metabolic switches between lanthanum (La)- and Ca-dependent growth. The gene expression study conducted by Larsen and Karlsen (2016) highlighted additional copper-linked regulatory switches in *M. capsulatus*. A total of 137 genes related to energy and transport metabolism were found to be differentially expressed between cells producing sMMO and pMMO. The study led to the detection of novel *c*-type cytochromes linked to copper-limited growth.

Tavormina and coauthors employed global gene-expression profiling to characterize cellular responses to methane starvation and recovery in the deep-sea aerobic methanotroph *Methyloprofundus sedimenti* (Tavormina et al. 2017). High transcript levels of methane monooxygenase genes and genes related to methanol utilization and lower transcript levels for other metabolic and housekeeping genes were demonstrated under active growth, while significant reduction of their expression including transcripts encoding methanol dehydrogenases (*mxa* and *xox*) was observed during starvation with one notable exception—transcript abundances for genes coding for methane monooxygenases increased considerably during starvation, but more notably, the *pmo* transcript abundance decreased during the early stage of recovery after methane starvation. Very similar responses have been found in numerous metatranscriptomic datasets, indicating significant metabolic bottlenecks for in situ methane utilization.

7.2.3 Proteomics

In order to discover microbial proteome profiles, high-resolution two-dimensional gel electrophoresis techniques and mass spectrometry approaches have been developed (Bensimon et al. 2012; Otto et al. 2014; Van Oudenhove and Devreese 2013). The data provide key insight into enzyme representation at the whole cell level and highlight posttranslational regulation of metabolic fluxes under different environmental growth conditions. Despite the advances in quantitative proteomics, however, there are not many examples of ubiquitous application of the approach to methanotrophy (Berven et al. 2006; Crombie and Murrell 2014; Gourion et al. 2006; Kao et al. 2004; Laukel et al. 2004). The most obvious cause is due to the structural complexity of the intracytoplasmic membrane that occupies a large portion of the cell volume and contains most of the essential proteins for the initial steps of assimilation pathways

(Best and Higgins 1981; Semrau et al. 2010). However, Berven et al. (2006) were able to analyze the outer membrane subproteome of *M. capsulatus* (Bath). Twenty-eight unique polypeptides were identified from proteins enriched in the outer membrane using two-dimensional gel electrophoresis coupled with electrospray ionization mass spectrometry. Of these, only the location and function of six of the polypeptides were previously known. Bioinformatics allowed predictions to be made for the functions of many of the previously unidentified proteins (β-barrel outer membrane proteins, lipoproteins, or cell surface proteins) that were in very good agreement with experimental data. In addition to this study for *M. capsulatus* (Bath), Kao et al. (2004) conducted a comprehensive quantitative analysis of the methanotroph's proteome for cells grown in the presence of different copper ion concentrations. Combining growth-limiting experiments with copper further led to interesting new discoveries such as the presence of all the genes for the serine cycle as well as key differences in expression between copper-starved and control bacteria at key metabolic enzymes such as formylmethanofuran hydrolase and many of the first or second enzymes in the C_1 assimilatory pathways (serine pathway, TCA, and RuMP) (Kao et al. 2004). Similar effects on transcriptional regulation of oxidative enzymes have been shown with MDH and the ratio of lanthanides to calcium in other methanotrophs (Chu and Lidstrom 2016; Haque et al. 2015). The most recent example of the application of proteomics is a study in which the ability of a single bacterial strain, *Methylocella silvestris*, to grow on methane or short-chain alkane was evaluated. The underlying mechanisms by which the methanotrophic strain used methane or propane as a carbon and energy source was determined (Crombie and Murrell 2014).

7.2.4 Metabolomics

In the context of systems biology, metabolomic approaches for the comprehensive identification and the accurate quantification of metabolites are now regarded as a valuable asset for protein or transcript profiling (Yang et al. 2012). Both targeted and global nontargeted approaches have been applied to investigate metabolic pathways in methanotrophic bacteria (Akberdin et al. 2018; Yang et al. 2013). A dynamic flux analysis based on ^{13}C metabolomics technology was the basis for the quantitative determination of a novel ethylmalonyl-CoA (EMC) pathway as an essential component for glyoxylate regeneration in *M. trichosporium* OB3b (Yang et al. 2013). A similar dynamic approach allowed the demonstration of the propensity for *Methylomicrobium alcaliphilum* 20Z to employ a pyrophosphate-mediated EMP variant of the RuMP pathway as the main route for C_1-assimilation under oxygen-limiting conditions (Kalyuzhnaya et al. 2013). ^{13}C-carbon tracings have determined the complete oxidative TCA cycle in *M. buryantense* (Fu et al. 2017). It should be mentioned that despite considerable advances in metabolomics technologies, many critical limitations must be considered, such as the leakage of intracellular metabolites into the solution, the overlap of many compartmentalized metabolic processes in the cell, and the complications linked to the interpretation of ^{13}C single carbon-labeling patterns.

7.3 Metabolic Modeling of Methane Metabolism

Currently, mathematical modeling approaches have become a basic framework for the integration and analysis of experimental data and the iterative investigation of dynamic biological systems (Akberdin et al. 2013; Hübner et al. 2011; Mast et al 2014; Sanchez-Osorio et al. 2014). The general type of model is determined on the basis of the available information, the use of qualitative or quantitative data, and the problem to solve. In a broad sense, the modeling approach as a key component of systems biology is becoming a standard tool for theoretical interpretation of biological systems and prediction of novel genes and their functions.

To build a metabolic model, it is necessary to have a reconstruction of the metabolism for the organism of interest. A number of genome-scale biochemical network reconstructions of biotechnology-relevant methanotrophic bacteria are available in BioCyc (http://www.biocyc.org; Caspi et al. 2016) or in the more commonly referred KEGG databases (http://www.genome.jp/kegg/; Kanehisa and Goto 2000). However, they are based on automatic reconstructions, which should be carefully evaluated in accordance with published data for the microbe of interest (substrate consumption and biomass accumulation rates, biomass composition analysis, metabolic pathway validation via enzymatic activity, gene/protein expression, etc.) and converted into a mathematical model that can be analyzed through constraint-based linear programming approaches, such as COBRA (http://opencobra.sourceforge.net/openCOBRA/Welcome.html; Schellenberger et al. 2011) or Pathway Tools (http://bioinformatics.ai.sri.com/ptools/; Karp et al. 2015), at a global systems level and through nonlinear kinetic modeling at a more local mechanistic level. In ideal situations, the reconstruction should be further validated through comparison of model predictions to phenotypic data. Eventually, the metabolic modeling approach provides a scaffold for the integration and analysis of high throughput data such as transcriptomics, proteomics, and metabolomics.

A few metabolic models focused on CH_4 metabolism have been constructed (Table 7.2). The computational interpretation of this C_1-network has been initiated with a steady-state model of the central metabolism of the facultative methylotrophs *Methylobacterium extorquens* AM1 and *Methylobacillus flagellatum* KT (Van Dien and Lidstrom 2002). The computational model has been further improved by implementation of a ^{13}C-fluxomics technique that was also applied to measure the distribution of metabolic fluxes under methanol growth conditions (Peyraud et al. 2011). The network-level analysis of the model indicated that the C_1-metabolic core in the methanotroph has a mosaic structure of embedded biochemical cycles. At the same time, it was demonstrated that multiple genes, which encode essential enzymes for methanol assimilation, are not functionally redundant, thereby explaining the structural fragility of the system. It has been concluded that the entire metabolism of the C_1-utilization is redox limited (Leak and Dalton 1983; Sipkema et al. 2000; Yoon and Semra 2008). However, contrary to methylotrophic models, the theoretical calculation of methanotrophy showed very poor correlation with measured parameters (Leak and Dalton 1986a). Critical factors that continue to hinder the development of computational models of methane utilization include the lack of

Table 7.2 Metabolic models for aerobic methane metabolism

Species	Model ID	Description
Methylosinus trichosporium OB3b	no ID (Sipkema et al. 2000)	Reactions, 7; Metabolites, 11 metabolic model developed for *Methylosinus trichosporium* OB3b The metabolic model presents a set of ordinary differential equations (time-dependent mass balances) and describes growth of *M. trichosporium* OB3b on methane in a continuous culture at various dilution rates and the metabolic responses of the organism to pulses of the intermediates methanol, formaldehyde, and formate. Validated by comparing experimental data (batch and transient-state measurements) with model simulations using the standard set of parameters. In silico predicted concentration of PHB (poly-α-hydroxybutyric acid) was matched to fluorometrically determined data.
Methylomicrobium buryatense 5G(B1)	*i*Mb5G(B1) (Torre et al. 2015) http://sci.sdsu.edu/kalyuzhlab	Reactions, 841; Metabolites, 1029 The genome-scale metabolic model (GEM) was constructed on basis of the whole-genome sequence and incorporates two types of MMO for methane oxidation, H4MPT and H4F pathways for formaldehyde oxidation and RuMP, EDD, EMP, and bifidobacterial shunt pathways for pyruvate biosynthesis, partial serine cycle, and TCA cycle with nonfunctional α-ketoglutarate dehydrogenase. Validated by biomass composition measurements.
Methylomicrobium alcaliphilum 20Z	*i*IA332 (Akberdin et al. 2018) http://sci.sdsu.edu/kalyuzhlab	Reactions, 431; Metabolites, 422 According to genomic and transcriptomic data for the strain, the GEM consists of pMMO for methane oxidation, H_4MPT and H_4F pathways for formaldehyde oxidation and RuMP, EDD, EMP, and bifidobacterial shunt pathways that are central pathways for pyruvate biosynthesis, partial serine cycle, TCA cycle with nonfunctional α-ketoglutarate dehydrogenase, and alternative TCA route via succinate-semialdehyde dehydrogenase+ additional aspartate loop through aspartate lyase; reverse phosphoketolase reaction for xylulose-5-phosphate. Validated by transcriptomic data, nontargeted metabolomic profiling + enzyme activity assay.

(continued)

Table 7.2 (continued)

Species	Model ID	Description
Methylococcus capsulatus	*i*CL656 (Lieven et al. in preparation)	Reactions, 726; Metabolites, 807 The model includes all major biosynthetic pathways for amino acids, cell wall components, fatty acids, membrane lipids and cofactors; detailed representation of the respiratory chain, the RuMP pathway, the nitrogen metabolism (assimilation and interconversions). Validated by measurements of growth rate in different copper-dependent conditions and carbon conversion efficiency.

fundamental knowledge of the initial steps of methane metabolism, from the catalytic mechanism of methane activation to the structural organization of the methane oxidation apparatus in biological systems.

To address these challenges, the first stoichiometric flux balance model of *Methylomicrobium buryatense* strain 5G(B1) has been constructed and used for evaluating different metabolic arrangements of methane oxidation and assimilation (Torre et al. 2015). Three arrangements were considered for methane oxidation: *redox mode*, the currently accepted model in which electrons driving methane oxidation come from NADH produced by formate or formaldehyde oxidation, while electrons produced from methanol oxidation are linked to the *redox* and used for ATP production; the *direct coupling mode*, in which methanol oxidation supplies electrons for methane oxidation without any additional inputs; and, finally, the *uphill electron transfer mode*, in which electrons driving methane oxidation come from cytochrome c to ubiquinone. The model simulations suggested the *direct coupling mode* is the most compelling mode of methane oxidation, and only this arrangement can support measured growth parameters, while the scenario employing NADH as a possible source of electrons for particulate methane monooxygenase cannot. Recently a developed genome-scale model for a closely related species, *Methylomicrobium alcaliphilum* $20Z^R$, has highlighted the dynamic behavior of methane oxidation machinery (Akberdin et al. 2018) and indicated the necessity of an additional constraint on the O_2 consumption rate to correctly reproduce experimentally observed parameters (growth rate and corresponding yields). The flux balance analysis of the model combined with global, nontargeted, metabolomic profiling and enzymatic assays highlighted the importance of the substitution of ATP-linked steps with PPi-dependent reactions and supported the presence of a carbon shunt from acetyl-CoA to the pentose-phosphate pathway and highly branched TCA cycle (Akberdin et al. 2018).

A genome-scale metabolic model of *Methylococcus capsulatus*, tentatively termed iCL656, has been constructed by extending and curating an automatically generated draft reconstruction published in 2012 as part of the Path2Models project (Büchel et al. 2013). As a genome-scale metabolic model, iCL656 includes all major

biosynthetic pathways for amino acids, cell-wall components, fatty acids, membrane lipids, and cofactors. In addition, the presence of a detailed representation of the respiratory chain, the RuMP pathway, and the nitrogen metabolism (assimilation and interconversions) provide a comprehensive insight into the metabolism of *M. capsulatus*. The model's predictions of growth yields and O_2/CH_4 ratios agree well with an experimental dataset published by Leak and Dalton (1986a) and indicate that like *Methylomicrobium* species, *M. capsulatus* may also use electrons from a methanol oxidation step.

In the most recent development, a kinetic modeling approach that accounts for systems dynamics at the metabolite level as well as regulatory effects has been applied (Akberdin et al. n.d.). Kinetic models are particularly suitable to the study of metabolic systems (Karr et al. 2012; Kitano 2001; Klipp et al. 2008) because they are capable of representing the complex biochemistry of cells in a more complete way compared to other types of models and provide quantitative predictions of the system in response to different inputs. To decipher the puzzle of electron transfer system in methanotrophs, the first kinetic model was recently constructed for *Methylomicrobium alcaliphilum* $20Z^R$ (Akberdin et al. 2018). Model analysis combined with a mutagenesis study on components of the electron transport chain demonstrates that direct coupling is the most compelling mode of the methane oxidation in the steady state, while NADH is essential for the initial activation of pMMO upon substrate limitation.

7.4 Final Remarks

Overall, the metabolic reconstruction of the methane metabolic network coupled with systems-biology approaches has greatly advanced our understanding of methane utilization and highlighted the importance of further investigation of the initial steps of methane utilization. The redundancy of methane and methanol oxidation machineries and the importance of iron, copper, and lanthanum in governing the switch between the key enzymes also await a thorough investigation. We should also expect advances in metabolic modeling of Alphaproteobacterial and Verrucomicrobial systems, as well as descriptions of metabolic interplays between methanotrophic and non-methanotrophic bacteria in complex microbial communities.

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