Marina G. Kalyuzhnaya · Xin-Hui Xing
Editors

Methane Biocatalysis: Paving the Way to Sustainability

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ISBN 978-3-319-74865-8 ISBN 978-3-319-74866-5 (eBook)
https://doi.org/10.1007/978-3-319-74866-5

Library of Congress Control Number: 2018940194

Printed on acid-free paper

This Springer imprint is published by the registered company Springer International Publishing AG part of Springer Nature.
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

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Methanotrophy: An Evolving Field

1

Ludmila Chistoserdova

As a field, methanotrophy has emerged in the early twentieth century, marked by the discovery of microbes that could sustain growth on methane gas, using it as the source of both carbon and energy. One hundred plus years later, the field is mature, having accumulated deep knowledge on different modes of methane metabolism, in microbes of different domains of life, bacteria and archaea, both aerobic and anaerobic. The past decade in methanotrophy has been marked by new important discoveries, including novel guilds of methanotrophs, novel metabolic modes, and novel enzymes and pathways, demonstrating that methanotrophy is an evolving field, and, likely, much is yet to be discovered. Future challenges include deciphering the mechanistic details of methane activation by the particulate methane monooxygenase, including the source of electrons in this reaction, understanding the respective functions of redundant enzymes such as alternative methane monooxygenases, methanol dehydrogenases, and other enzymes and pathways, and obtaining further insights into the evolution of methanotrophy, both aerobic and anaerobic. While methane is practically unlimited on this planet, thus presenting an attractive, renewable source of carbon for biotechnological use, including synthesis of fuels, multiple technical challenges exist in harnessing extant methanotrophs as efficient commercial platforms or, reversely, in engineering established platforms, such as *E. coli* or yeast, to utilize carbon from methane.

1.1 A Brief History of Methanotrophy

Methanotrophy is a field of study focused on metabolism of methane, carried out by microorganisms, and it is over 100 years old. Discovery of methanotrophy as a metabolic mode can be dated to circa 1906, when papers were published describing

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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_1

microbes capable of growth on methane gas (Kaserer 1906; Söhngen 1906). The active exploration of the properties and the metabolic details of methanotrophy mostly date to the early 1970s, after Whittenbury and colleagues demonstrated that multiple cultures of methanotrophs can be isolated from a variety of environments, also describing media that support their growth in laboratory (Whittenbury et al. 1970). The protocol described by Whittenbury and the nitrate and ammonium minimal salts (NMS, AMS) media are still in use today (Dedysh and Dunfield 2017). Thin section microscopy of methanotrophic microbes revealed that their cells were peculiar in a way that they were filled with regular structures, recognized as internal membranes (Proctor et al. 1969), their presence appearing to be connected to methane-oxidizing activity (Anthony 1982). Moreover, two types of membrane structures were recognized, stacked, and peripherally distributed, suggesting that at least two different types of methanotrophs existed (Whittenbury et al. 1970). These types are now known, respectively, as gammaproteobacterial and alphaproteobacterial methanotrophs.

Insights into the biochemical pathways that enabled assimilation of methane carbon into biomass revealed that the differences between the two types of methanotrophs extended beyond membrane types and into central metabolism: the microbes possessing the stacked membranes assimilated carbon through condensation of formaldehyde with a sugar molecule, through the ribulose monophosphate (RuMP) cycle, and microbes with the peripheral membranes condensed formaldehyde with an amino acid, through the serine cycle. These different types are still referred to as Type I and Type II methanotrophs, respectively (Trotsenko and Murrell 2008). Early studies of the main enzyme in methanotrophy, the one that activates the highly inert molecule of methane, produced some controversial results, one group identifying a soluble multisubunit enzyme (Colby and Dalton 1978), another identifying a membrane-bound enzyme, whose subunits had different molecular masses (Tonge et al. 1977). The controversy was later solved by the realization that both forms exist, the soluble (sMMO) and the particulate (pMMO) methane monooxygenases, and these days, multiple structure solutions have been generated for both enzymes (Ross and Rosenzweig 2017). While the ultimate source of electrons for the sMMO is NADH, the source of electrons for pMMO or the exact mechanism of methane activation by this enzyme remain undefined (Ross and Rosenzweig 2017).

Downstream of methane, the enzyme responsible for oxidation of methanol, the pyrroloquinoline quinone (PQQ)-linked methanol dehydrogenase, has also been thoroughly analyzed (Anthony and Zatman 1964, 1965, 1967a, b), and this enzyme and the respective genes have been found highly conserved among Type I and Type II methanotrophs, as well as among non-methanotrophic methylotrophs (Lidstrom et al. 1994).

Enzymes/pathways for formaldehyde oxidation have also been analyzed, identifying multiple possible candidates, which included the dissimilatory RuMP cycle, the glutathione-linked formaldehyde oxidation pathway, the tetrahydrofolate (H_4F)-linked pathway, as well as putative NAD-linked and dye-linked formaldehyde dehydrogenases (Anthony 1982). As a rather surprising addition to all these potential

pathways for formaldehyde oxidation, a pathway has been uncovered in the late 1990s, involving reactions dependent on tetrahydromethanopterin (H_4MPT) and methanofuran (MF; Chistoserdova et al. 1998), previously characterized in anaerobic methanogenic archaea (Thauer 1998), and this pathway has been demonstrated to be widely distributed among different guilds of methylotrophs (Vorholt et al. 1999). The discovery of this pathway not only expanded the understanding of the metabolic potential of aerobic methylotrophs but also questioned the evolution of their metabolism, raising questions of how the same or very similar enzymes could carry out reactions key to "strictly aerobic" and "strictly anaerobic" metabolisms (Chistoserdova et al. 1998, 2004).

The details of the metabolic transformations constituting both the RuMP and the serine cycles were mainly deciphered by Quayle and colleagues, in series of studies simple in their elegance, mostly using labeling with radioactive carbon from methanol, formate or CO_2, followed by chromatographic analysis of the metabolites (Large et al. 1961, 1962a, b; Large and Quayle 1963; Kemp and Quayle 1965, 1966, 1967; Salem et al. 1972; Strøm et al. 1974). While modern approaches such as metabolomics and flux analysis can now be applied to precisely model carbon flux distribution among different reactions (Peyraud et al. 2009; Kalyuzhnaya et al. 2013; de la Torre et al. 2015), the pathways as outlined in the 1960s and 1970s remain true today, with perhaps one exception. The ethylmalonyl-CoA (EMC) pathway, a pathway for conversion of acetyl-CoA, functioning as part of the serine cycle, has remained a mystery for about 50 years since the deficiency of some of the serine cycle methylotrophs in the glyoxylate cycle has been discovered (Anthony 2011), being completely resolved only between 2007 and 2009 (Erb et al. 2007, 2009). While it remains unknown why some methylotrophs use the glyoxylate cycle, some use the EMC pathway, and some use both for either methylotrophy or acetate metabolism (Chistoserdova 2011); a similar situation exists in archaea, some of which utilize the glyoxylate cycle and some utilize an alternative methylaspartate cycle, which, in turn, shares some of the reactions with the EMC pathway (Khomyakova et al. 2011).

The process of anaerobic oxidation of methane (AOM) has also been known for a long time, based on the geochemical evidence (Reeburgh 1976, 1980). The microbes involved in this process were identified relatively recently, and these were found to be archaea and not bacteria (known as ANME-type archaea) (Hinrichs et al. 1999; Boetius et al. 2000; Orphan et al. 2001; Knittel and Boetius 2009). The early metagenomics studies suggested that methanotrophy must be carried out by these species using a reverse methanogenesis pathway (Hallam et al. 2004), which, with the exception of the early reactions transforming methane into a methyl moiety attached to coenzyme M (Scheller et al. 2010), would be similar to the oxidation of formaldehyde carried out through H_4MT and MF-linked reactions by the bacterial methanotrophs (Chistoserdova et al. 2004). Methane carbon was proposed to be assimilated via the Wood-Ljungdahl pathway (Hallam et al. 2004) that is also used by both the methanogenic archaea and the anaerobic methylotrophic clostridia (Drake et al. 2008). Thus, while the aerobic and the anaerobic modes of methane oxidation were considered fundamentally different, they both involved several

common reactions and cofactors, again questioning the commonality of their evolution (Braakman and Smith 2012; Weiss et al. 2016).

1.2 The Past Decade in the Methanotrophy Field

While progress in methanotrophy research has been steady over the past 50 years or so, it seems to have especially accelerated over the past decade (Fig. 1). This acceleration may be partly attributed to the wide application of modern tools such as genomics and other systems approaches (Chistoserdova 2017) and partly perhaps to the renewed interest toward methanotrophs as prospective targets for biotechnological platform development (Kalyuzhnaya et al. 2015; Strong et al. 2015). The past 10 or so years saw discovery of novel guilds of methanotrophs, as well as of novel enzymes and pathways, along with several corrections to the metabolic modes characterized in the past. A brief review of these recent and exciting discoveries is presented below.

Two new guilds of methanotrophs have been recently discovered within the bacterial domain of life, phylogenetically distinct from the proteobacterial methanotrophs and belonging to *Verrucomicrobia* (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Op den Camp et al. 2009) and to the candidate phylum NC10 (Raghoebarsing et al. 2006; Ettwig et al. 2010). These discoveries likely suggest that methanotrophy may be occurring within the bacterial domain of life even more widely and new methanotroph species are awaiting to be discovered. Despite the long phylogenetic distances between *Proteobacteria*, *Verrucomicrobia*, and NC10 bacteria, the metabolic scheme for methane oxidation in the newly discovered guilds is similar to the one in *Proteobacteria*, and it proceeds all the way to CO_2, which is then assimilated via the classical Calvin-Benson-Bassham cycle (Khadem et al. 2011; Rasigraf et al. 2014), presenting a novel combination of the assimilatory/dissimilatory modules enabling methanotrophy (Chistoserdova 2011).

A novel methanol dehydrogenase (MDH) has also been discovered. While a gene, named *xoxF*, along with the respective protein XoxF, has puzzled the methylotrophy community for a long time (Chistoserdova 2011), and while evidence was available for this enzyme to have a function in methylotrophy (Mustakhimov et al. 2013), low activity with methanol (Schmidt et al. 2010) continued to suggest that something was amiss. The missing factor turned out to be rare Earth elements (REEs), playing a catalytic role in XoxF enzymes (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012), instead of calcium, the cofactor for the classic, MxaFI MDH enzyme (Anthony 2004). The hint on the catalytic role of REEs came from outside of the methylotrophy field (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012), and their potential significance has not been embraced right away. However, in the recent few years, the research in REE-dependent methanol oxidation has been exploding, demonstrating, in a variety of key model organisms, that not only REEs are involved in methanol catalysis but that they are also involved in inverse regulation of genes for alternative MDH enzymes (Pol et al. 2014; Vu et al.

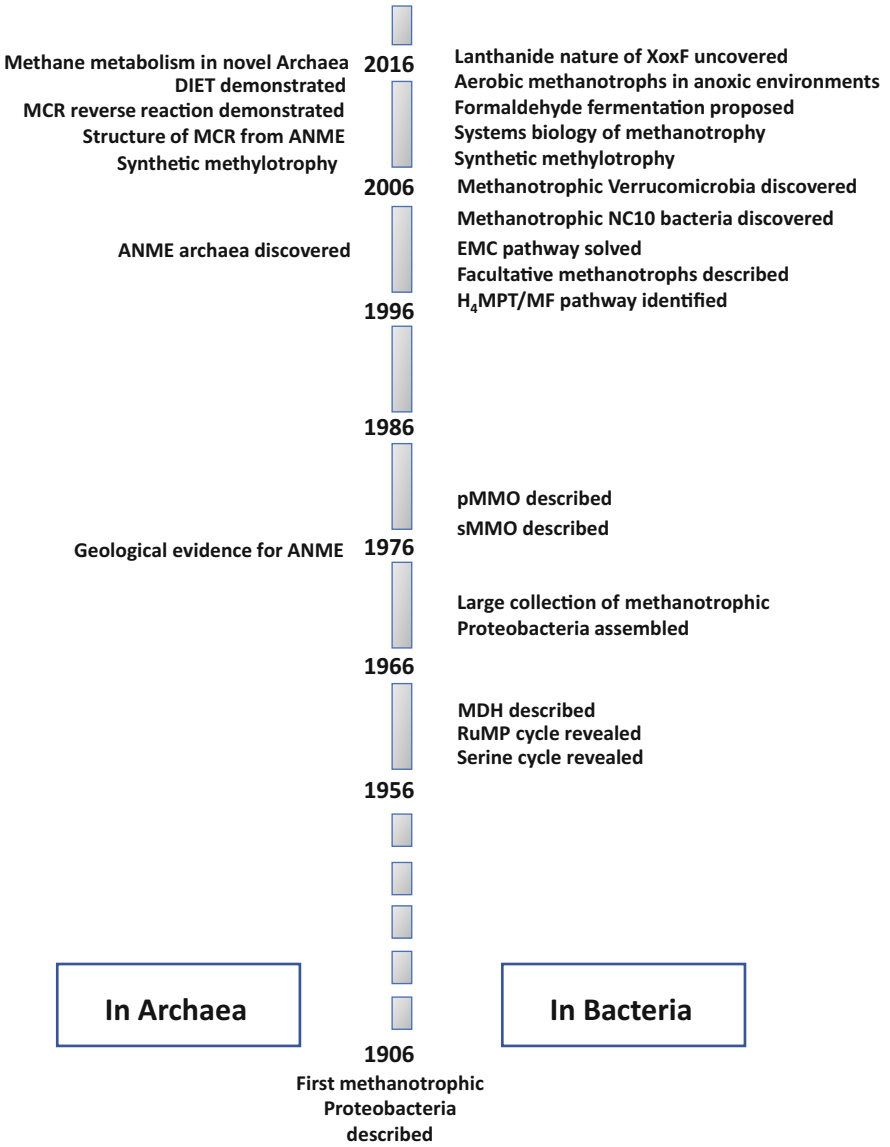

Fig. 1.1 Schematic of timeline for landmark discoveries in methanotrophy

2016; Chu and Lidstrom 2016, Chu et al. 2016; Gu et al. 2016). Moreover, XoxF-type MDH appears to be more environmentally widespread and more divergent than MxaFI enzymes, suggesting its ancestral origin (Chistoserdova 2011, 2015, 2017; Keltjens et al. 2014). The verrucomicrobial methanotrophs so far appear to only encode XoxF (Pol et al. 2014), the NC10 methanotrophs encode both MxaFI and XoxF (Ettwig et al. 2010), and proteobacterial methanotrophs encode either both

enzymes or only XoxF (Chistoserdova 2011; Vekeman et al. 2016; Padilla et al. 2017).

The major adjustments to the known methanotrophy pathways included the EMC pathway, as mentioned above, which changed the accepted balance between carbon from methane versus CO_2 carbon assimilated by these microbes (Anthony 1982), from 2:1 to 1:1 (Peyraud et al. 2009; Chistoserdova et al. 2009), highlighting the potential for these microbes in sequestering CO_2. Ironically, exactly this ratio was experimentally measured by the Quayle group in the 1960s (Large et al. 1961). The understanding of the metabolism of the RuMP cycle methanotrophs has also been adjusted to the original proposal by the Quayle group (Strøm et al. 1974), by uncovering that the glycolysis pathway is part of the RuMP cycle, along with the Entner-Doudoroff pathway (Kalyuzhnaya et al. 2013). Moreover, a formaldehyde fermentation pathway has been proposed utilizing reactions of the glycolysis pathway, as a metabolic mode for conditions of limited oxygen (Kalyuzhnaya et al. 2013).

Of the other dogmas established in the past century, the dogma of "obligate" methanotrophy, first questioned in 2005 (Dedysh et al. 2005), has been further dismantled, as least for the alphaproteobacterial methanotrophs (Semrau et al. 2011; Crombie and Murrell 2014; Dunfield and Dedysh 2014). In the gammaproteobacterial methanotrophs, the operation of the complete citric acid cycle, in the classic oxidative direction, has also been demonstrated (Fu et al. 2017). One of the most intriguing recent observations on "aerobic" methanotrophs that deviates from the doctrine is the apparent propensity of "aerobic" methanotrophs, especially representatives of the genus *Methylobacter*, to thrive in anoxic environments (Martineau et al. 2010; Graef et al. 2011; Tveit et al. 2013, 2014; Blees et al. 2014; Crevecoeur et al. 2015; Osvald et al. 2015, 2016a,b; Padilla et al. 2017; Martinez-Cruz et al. 2017). A denitrification capability has been uncovered in both proteobacterial methanotrophs and methanotrophs of the NC10 phylum, suggesting alternative electron acceptors (Ettwig et al. 2010; Kits et al. 2015). In the case of NC10 bacteria, a novel mechanism for intracellular O_2 production has also been proposed (Ettwig et al. 2010). However, activity of "aerobic" methanotrophs has been demonstrated in environments devoid of nitrate/nitrite (Milucka et al. 2015), suggesting alternative metabolic scenarios. It has been proposed recently that cryptic oxygen cycling is common in seemingly anoxic environments due to tight coupling of oxygen production and consumption, thus keeping oxygen at levels as low as subnanomolar (Garcia-Robledo et al. 2017).

Significant progress has been also made in understanding methane oxidation by the archaea. Reverse reaction activity for methyl-CoM reductase (MCR) has been experimentally demonstrated, supporting the role of this enzyme in primary methane oxidation by archaea (Scheller et al. 2010). In further support, the MCR homolog from a microbial mat active in methane oxidation revealed striking structural similarities with MCR enzymes involved in methanogenesis (Shima et al. 2011), providing firm evidence that methane production and methane oxidation must rely on the same enzyme. Moreover, it has even been demonstrated that ANME-type archaea can both produce and oxidize methane; this conclusion based on

quantification of gene transcripts of ANME in zones of methane oxidation and methane production, separated across the depths of a sediment (Lloyd et al. 2011).

Further progress has also been made toward resolving the potential mechanisms for interspecies electron transfer that is essential for anaerobic methane oxidation (Boetius et al. 2000; Orphan et al. 2001). The latest proposals favor direct electron transfer (DIET) between ANME and sulfate-reducing bacteria, which is mediated by pili as well as by multiheme cytochromes (McGlynn et al. 2015; Wegener et al. 2015; Krukenberg et al. 2016). Further support for DIET was obtained through decoupling AOM from sulfate reduction using artificial electron acceptors (Scheller et al. 2016). Methane oxidation by ANME linked to denitrification has also been discovered (Haroon et al. 2013; Arshad et al. 2015), this metabolism also involving a syntrophic partner, the anaerobic ammonia-oxidizing bacteria (Haroon et al. 2013). Moreover, novel lineages of archaea have been recently identified through culture-independent experiments with a potential in methane metabolism, belonging to novel phyla, Thorarchaeota (Seitz et al. 2016), Bathyarchaeota (Evans et al. 2015; Mwirichia et al. 2016; Lazar et al. 2016), and candidate phylum Verstraetearchaeota (Vanwonterghem et al. 2016). It remains to be demonstrated whether these novel organisms are active in methane oxidation, methanogenesis, or both.

Overall, discovery of novel phyla within both bacteria and archaea capable of methane transformations, including species possessing H_4MPT/MF functions not yet assigned to any specific metabolic pathway, further suggests the common evolutionary history for methanotrophy and methanogenesis (Chistoserdova 2013, 2016) and the ancient nature of these reactions (Weiss et al. 2016).

Another concept in methanotrophy that received recent support is the communal nature of the microbial metabolism of methane. The syntrophic nature of anaerobic methane oxidation by the archaea has been recognized from the very start, supported by bioenergetic constraints of this process (Thauer and Shima 2008). The anaerobic NC10 bacteria appear to also be syntrophic, as they still have not been cultivated in pure form. However, while the proteobacterial methanotrophs can be cultivated in pure cultures, they as well tend to form consortia with other, non-methanotrophic organisms (Dedysh and Dunfield 2017). Moreover, recent experiments questioning the composition of such consortia have identified co-occurrence patterns suggesting some type of specificity in methanotroph/non-methanotroph associations (Hernandez et al. 2015; Oshkin et al. 2015). While some potential metabolic linkages have been identified such as sharing of methanol (Krause et al. 2017; Tavormina et al. 2017), whether these are guild-level or species-/strain-level linkages remains to be determined.

1.3 Future Challenges

The field of methanotrophy has come of age, having accumulated sophisticated knowledge on the details of both oxygen-dependent and oxygen-independent microbial processes converting methane into energy and biomass. Yet the past decade in methanotrophy has been marked by series of new and exciting discoveries that

identify novel directions in methanotrophy and also pose novel challenges to be addressed in the future. Some of these are of academic value, and some are relevant to the potential industrial applications of methanotrophs. In terms of the former, the details of the activation of methane by the pMMO remain to be uncovered, along with the source(s) of electrons for the activation. This problem has become even more profound in the light of the recent evidence of the activity of "aerobic" methanotrophs in anoxic environments. Thus, along with the catalytic mechanisms, questions need to be resolved of how oxygen molecules are being sensed, accessed, stored, and transferred to the pMMO. Are the electrons involved in enzyme activation coming from within or from outside the cell, and can pMMO switch between different sources of electrons? Are there similarities between "aerobic" and "anaerobic" methanotrophs with this respect? Interestingly, multiheme cytochromes, akin to the cytochromes characterized in electricity-generating bacteria (Lovley 2017) or in ANME-type methane oxidizers (McGlynn et al. 2015; Wegener et al. 2015; Krukenberg et al. 2016), have been identified in some aerobic methanotrophs (Karlsen et al. 2011). Another question that still remains is whether sMMO and pMMO are redundant or whether they are tailored to specific metabolic goals. Likewise, the history and the distinct functions of the alternative methanol dehydrogenases (XoxF vs. MxaFI) in methanotrophy need to be further addressed, as well as the role of REEs in methane oxidation. So far, the published research on lanthanides in methylotrophy has used unnaturally high concentrations of REEs (Hibi et al. 2011; Fitriyanto et al. 2011; Nakagawa et al. 2012; Pol et al. 2014; Vu et al. 2016; Chu and Lidstrom 2016, Chu et al. 2016; Gu et al. 2016), which in turn, resulted in quick selection of mutants with modified behavior with relation to REEs (Chu et al. 2016). However, natural concentrations of REEs belong in a dramatically different range (Amyot et al. 2017; Turetta et al. 2017), posing questions whether, instead of the so-called lanthanide switch (Chu et al. 2016), a fine-tuned synergy exists between XoxF-type and MxaFI-type MDH enzymes and whether methanotroph communities compete for or share REEs. The questions about the evolution of methanotrophy are also becoming more intriguing as the recent data present more possibilities, given the facts that the "aerobic" methanotrophs are not so aerobic after all (Danilova et al. 2016), that common pathways are widespread among different guilds of "aerobic" and "anaerobic" methanotrophs, and that autotrophy now appears rather common in methanotrophy. The role of syntrophies in "aerobic" methane oxidation needs to be further questioned, in experiments with natural as well as synthetic communities, which may present novel models for studying methanotrophy (Yu and Chistoserdova 2017).

Nowadays, the significance of methane as a carbon source that could be utilized by the modern humanity has been increasing, considering that methane is practically unlimited on this planet, and that removal of methane, steadily produced by both natural and anthropogenic sources, and its conversion into value-added compounds, including fuels, would present the most practical solution to both greenhouse effect mitigation and to harvesting an abundant and sustainable carbon compound. While methanotrophs present attractive biotechnological platforms, at least theoretically (Kalyuzhnaya et al. 2015), many challenges exist that prevented their broad use on

large and commercially feasible scales (Strong et al. 2015). A reverse approach, of engineering some of the well-developed and commercially feasible platforms, such as *E. coli* or yeast, to consume methane with an output of value-added compounds, has also been challenging, especially in terms of integration of the methane oxidation module(s), and so far, such an approach lacks any evidence of a positive outcome. However, success was reported with engineering *E. coli* capable of converting methanol into value-added compounds (Whitaker et al. 2017). Success was also reported with engineering a methane-consuming recombinant archaeon, expressing archaeal methane oxidation module (Soo et al. 2016). Thus, both approaches, of engineering native methane oxidizers as well as recombinant strains, are worth pursuing in the future, as the new tools and technologies keep pushing the technical limitations and as, at the same time, the range of the organisms of potential commercial interest, including communities versus single cultures, is constantly broadening. Whether the methanotrophs (or methanotrophy) are ever fully harnessed in commercial applications, they will never cease to be an exciting group of organisms, possessing a unique capability of converting methane into biomass, aerobically or anaerobically.

Acknowledgments Support by the US Department of Energy (DE-SC-0016224) is acknowledged.

Conflict of Interest The author declares no conflicts of interest.

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Diversity and Phylogeny of Described Aerobic Methanotrophs

2

Svetlana N. Dedysh and Claudia Knief

2.1 Introduction

Aerobic methanotrophs are a unique subset of methylotrophic bacteria that can utilize methane (CH_4) as a sole energy source (Hanson and Hanson 1996; Trotsenko and Murrell 2008; Semrau et al. 2010). A defining characteristic of these organisms is the use of methane monooxygenase (MMO) enzymes to catalyze the oxidation of methane to methanol. MMO occurs in two forms, a membrane-bound or particulate (pMMO) and a soluble form (sMMO). Methanotrophic bacteria inhabit a wide range of habitats where both methane and oxygen are available (Hanson and Hanson 1996; Nazaries et al. 2013; Knief 2015).

The first methanotrophic bacterium was isolated by Söhngen and named "*Bacillus methanicus*" (now known as *Methylomonas methanica*) (Söhngen 1906). Since that time, the number and diversity of described methanotrophs has gradually increased. At present, methanotrophic capabilities relying on MMO activity are recognized in members of the bacterial phyla *Proteobacteria*, *Verrucomicrobia*, and the candidate division NC10 (Stein et al. 2012). Nearly all described methanotrophic bacteria that are now available in pure cultures belong to the *Proteobacteria*. These microorganisms affiliate with the classes *Gammaproteobacteria* (type I methanotrophs) and *Alphaproteobacteria* (type II methanotrophs). Methanotrophic *Verrucomicrobia* were only recently discovered (Op den Camp et al. 2009) and are represented by a limited number of isolates. Methanotrophic representatives of the candidate phylum NC10, "*Candidatus* Methylomirabilis oxyfera"-like methanotrophs, occur in anoxic habitats

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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_2

and have an intra-aerobic pathway of CH_4 oxidation (Ettwig et al. 2010). These bacteria have not yet been obtained in pure cultures and are not further discussed in this chapter.

The selective approach to enrich and cultivate methanotrophs implies the use of mineral media with methane as a growth substrate. The variety of media and the most common techniques used in methanotroph cultivation were recently reviewed by Dedysh and Dunfield (2014). Final steps of the isolation procedure include thorough examination of the methanotrophic cultures for purity, registration of their growth dynamics on methane, and molecular identification (see Dedysh and Dunfield 2011). The required tests as well as the minimal standards for characterization of novel aerobic methanotrophs are described by Bowman (2011).

2.2 Major Phylogenetic Groups of Aerobic Methanotrophs

Based on physiological, morphological, ultrastructural, and chemotaxonomic traits, all aerobic methanotrophs have been originally divided into two major groups, type I and type II methanotrophs (Whittenbury and Dalton 1991; Hanson and Hanson 1996). Major distinctive features between type I and type II methanotrophs were the carbon fixation mechanism via the ribulose monophosphate pathway (type I) or serine cycle (type II), the capability of nitrogen fixation, the arrangement of intracytoplasmic membranes (ICM) as vesicular discs (type I) or paired membranes aligned to the cell periphery (type II), and the predominance of specific C16 (type I) or C18 (type II) fatty acids. Phylogenetic analyses of 16S rRNA gene sequences confirmed this classification, whereby type I and type II methanotrophs affiliated with the *Gammaproteobacteria* and *Alphaproteobacteria*, respectively. Further extension of characterized methanotroph diversity, however, has turned the original distinction based on the abovementioned criteria largely into question. While the major carbon fixation pathway is still a distinctive feature, other characteristics are no longer exclusive for one or the other group (see Knief 2015). For example, members of the family *Methylothermaceae* (gammaproteobacterial methanotrophs) are characterized by the predominance of C18 fatty acids, and representatives of the genera *Methylocella* and *Methyloferula* (alphaproteobacterial methanotrophs) do not possess ICM. Because of these exceptions, the original concept of type I and II methanotrophs is no longer useful to categorize all known aerobic methanotrophic bacteria, and it has been proposed to abandon it (Op den Camp et al. 2009; Semrau et al. 2010). Nevertheless, the terms are still frequently used and have been adapted to the increasing diversity of methanotrophs during the last years. However, they should only be considered as synonyms for the phylogenetic groups of methanotrophic *Alpha-* and *Gammaproteobacteria*.

Nowadays, type I and sometimes also type II methanotrophs are divided into different subgroups (Fig. 2.1). For type I methanotrophs, this subgrouping is not consistent among all publications, but a common categorization divides the group of *Methylococcaceae* into types Ia and Ib, while the *Methylothermaceae* represent type Ic methanotrophs (Knief 2015). The differentiation into types Ia and Ib within the family *Methylococcaceae* corresponds to an earlier proposed grouping, in which

Methylococcus, *Methylocaldum*, and related genera (now type Ib) were already distinguished as type X methanotrophs based on physiological characteristics (Green 1992; Hanson and Hanson 1996; Bowman 2006). Type II methanotrophs are sometimes further differentiated into IIa and IIb according to their classification as *Methylocystaceae* and *Beijerinckiaceae*, respectively (Fig. 2.1). This separation into additional subgroups has no taxonomic meaning and is used mostly in molecular diversity studies.

2.3 Taxonomically Described Diversity

According to the taxonomic status, all currently described methanotrophic bacteria can be divided into three categories: (1) characterized methanotrophs with validly published names, (2) methanotrophs with tentative names, and (3) methanotrophs with a *Candidatus* status. With some exceptions (see comments on *Crenothrix polyspora* in Sect. 2.3.1), the first category is represented by the organisms that were isolated in pure cultures and comprehensively characterized (Table 2.1). Type strains of these species have been deposited in public culture collections. The names of these methanotrophs are included in the List of Prokaryotic names with Standing in Nomenclature (http://www.bacterio.net/) (Parte 2014). The second category includes those methanotrophs, which were also isolated in pure cultures but, due to some reasons, were either not deposited in public culture collections or only partly characterized. In some cases, the newly proposed names were simply never submitted for validation. The names of these organisms appear in quotations (e.g., "*Methylomonas denitrificans*"). Finally, the category *Candidatus* was established for certain putative taxa that could not be described in sufficient detail to warrant establishment of a novel taxon (http://www.bacterio.net/-candidatus.html). This category is commonly used for organisms that could not yet be isolated in pure cultures. In addition to genomic information such as sequences to determine the phylogenetic position of the organism, all information, including structural, metabolic, and reproductive features, should be included in the description, together with the natural environment in which the organism can be identified by in situ hybridization or other similar techniques for cell identification. The names included in the category *Candidatus* are usually written as follows: "*Candidatus* Methylospira mobilis." Below, we give an overview of the methanotrophic taxa representing these three categories.

2.3.1 Family *Methylococcaceae*

This family belongs to the class *Gammaproteobacteria*, the order *Methylococcales*, and accommodates Gram-negative, aerobic bacteria, which divide by binary fission and are restricted to methane and methanol as sole sources of carbon and energy (Bowman 2016a). Cells contain type I intracytoplasmic membranes appearing as stacks of vesicular discs. Methane is oxidized by pMMO; sMMO activity is rare (Table 2.1). C_1

Table 2.1 Currently described aerobic methanotrophic bacteria that were isolated in pure cultures

Genus	Species	pMMO/ sMMO	Specifics of physiology	Reference[a]
Class *Gammaproteobacteria*, order *Methylococcales*, family *Methylococcaceae*				
Methylococcus	*M. capsulatus*	+/+	Moderately thermophilic	Bowman (2015a)
	M. thermophilus	+/nd		
Methylomonas	*M. methanica*	+/v		Bowman (2016b)
	M. aurantiaca	+/nd		
	M. fodinarum	+/nd		
	M. koyamae	+/−		
	M. scandinavica	+/−	Psychrotolerant	
	M. lenta	+/−		
	M. paludis	+/−	Mildly acidophilic	
	M. denitrificans	+/−		
Methylobacter	*M. luteus*	+/−		Kalyuzhnaya (2017)
	M. marinus	+/−	Slightly halophilic	
	M. whittenburyi	+/−		
	M. tundripaludum	+/−	Psychrotolerant	
	M. psychrophilus	+/−	Psychrophilic	
	M. modestohalophilus	+/−	Moderately halophilic	
Methylomicrobium	*M. agile*	+/−		Kalyuzhnaya (2016a)
	M. album	+/−		
	M. alcaliphilum	+/−	Haloalkaliphilic	
	M. japanense	+/+		
	M. kenyense	+/−	Haloalkaliphilic	
	M. pelagicum	+/−		
	M. buryatense	+/v	Haloalkaliphilic	
Methylosarcina	*M. fibrata*	+/−	Mesophilic and neutrophilic; capable of growth in mildly acidic habitats	Kalyuzhnaya (2016b)
	M. quisquiliarum			
	M. lacus			
Methylocaldum	*M. gracile*	+/−	Thermotolerant and moderately thermophilic	Takeuchi (2016)
	M. marinum	+/+		
	M. szegediense	+/−		
	M. tepidum	+/−		
Methylogaea	*M. oryzae*	+/−	Mesophilic and neutrophilic	Tarlera (2016)
Methylosoma	*M. difficile*	+/−	Microaerobic	Schink and Rahalkar (2016)
Methyloparacoccus	*M. murrellii*	+/−		Hoefman et al. (2014)

(continued)

Table 2.1 (continued)

Genus	Species	pMMO/ sMMO	Specifics of physiology	Reference[a]
Methyloglobulus	*M. morosus*	+/−	Microaerobic	Schink and Deutzmann (2016)
Methyloprofundus	*M. sedimenti*	+/−	Psychrotolerant and slightly halophilic	Tavormina (2016)
Methylomarinum	*M. vadi*	+/−	Slightly halophilic	Hirayama (2016a)
Methylovulum	*M. miyakonense*	+/+		Iguchi et al. (2016)
	M. psychrotolerans	+/−	Psychrotolerant	Oshkin et al. (2016)
Methylomagnum	*M. ishizawai*	+/+		Khalifa et al. (2015)
Methylosphaera	*M. hansonii*	+/−	Psychrophilic	Bowman (2015b)
Class *Gammaproteobacteria*, order *Methylococcales*, family *Methylothermaceae*				
Methylothermus	*M. thermalis*	+/−	Moderately thermophilic	Hirayama (2016c)
	M. subterraneus			
Methylohalobius	*M. crimeensis*	+/−	Moderately halophilic	Dunfield (2016)
Methylomarinovum	*M. caldicuralii*	+/−	Moderately thermophilic	Hirayama (2016d)
Class *Alphaproteobacteria*, order *Rhizobiales*, family *Methylocystaceae*				
Methylosinus	*M. sporium*	+/+		Bowman (2015d)
	M. trichosporium	+/+		
Methylocystis	*M. parvus*	+/−		Bowman (2015c), Belova et al. (2013)
	M. echinoides	+/−		
	M. heyeri	+/+	Mildly acidophilic, facultatively methanotrophic, psychrotolerant	
	M. hirsuta	+/+		
	M. rosea	+/−	Psychrotolerant	
	M. bryophila	+/+	Mildly acidophilic, facultatively methanotrophic	
Class *Alphaproteobacteria*, order *Rhizobiales*, family *Beijerinckiaceae*				
Methylocella	*M. palustris*	−/+	Mildly acidophilic, psychrotolerant, facultatively methanotrophic	Dedysh and Dunfield (2016b)
	M. silvestris			
	M. tundrae			

(continued)

Table 2.1 (continued)

Genus	Species	pMMO/ sMMO	Specifics of physiology	Reference[a]
Methylocapsa	*M. acidiphila*	+/−	Mildly acidophilic, psychrotolerant	Dedysh (2016)
	M. aurea			
	M. palsarum			
Methyloferula	*M. stellata*	−/+	Mildly acidophilic, psychrotolerant	Dedysh and Dunfield (2016c)
Phylum *Verrucomicrobia*, order "*Methylacidophilales*," family "*Methylacidophilaceae*"				
"*Methylacidiphilum*"	"*M. infernorum*"	+/−	Acidophilic and thermophilic	Dunfield et al. (2007), Pol et al. (2007), Islam et al. (2008), Op den Camp et al. (2009)
	"*M. fumarolicum*"	+/−		
	"*M. kamchatkense*"	+/−		
"*Methylacidi-microbium*"	"*M. fagopyrum*"	+/−	Acidophilic	van Teeseling et al. (2014)
	"*M. cyclopophantes*"	+/−	Acidophilic	
	"*M. tartarophylax*"	+/−	Acidophilic	

[a]When available, the reference is given for the recently published chapter with the genus description in Bergey's Manual of Systematics of Archaea and Bacteria

compounds are assimilated via the ribulose monophosphate pathway. Some representatives grow best at low O_2 tensions.

The type genus of this family is *Methylococcus*. Cells of *Methylococcus* species appear as cocci or rods that occur singly, in pairs, and sometimes in chains (Bowman 2015b). Representatives with motile and nonmotile cells are known. C_1 compounds are assimilated via the ribulose monophosphate pathway; cells also contain a partially functional Benson–Calvin cycle. These methanotrophs fix dinitrogen via an oxygen-sensitive nitrogenase. They are thermotolerant or moderately thermophilic bacteria with optimal growth between 40 and 60 °C. Members of this genus were isolated from sediments of freshwater lakes and rivers, wetland muds, activated sludge, and wastewater.

Representatives of the genus *Methylomonas* are straight or slightly curved rods, occurring singly, in pairs, or in short chains. Most described species are motile by means of a single polar flagellum. These species often produce a surface pellicle in static liquid cultures. Production of red, pink, and orange carotenoid non-water-soluble pigments is highly typical for these methanotrophs. C_1 compounds are assimilated via the ribulose monophosphate pathway; ribulose-1,5-diphosphate carboxylase activity is absent. Some members of the genus can couple denitrification and methane oxidation. Several *Methylomonas* species fix dinitrogen via an oxygen-sensitive nitrogenase. Most representatives of the genus are mesophilic, growing between 10 and 40 °C. With the only exception of mildly acidophilic *M. paludis*, all

described species are neutrophilic. Habitats are sediments of freshwater lakes and rivers, wetland muds, activated sludge and wastewater, coal mine drainage waters, and groundwater (Bowman 2016b).

Cells of the majority of *Methylobacter* species possess a characteristic elliptical, rodlike morphology and occur singly, in pairs, or in chains. Cells are usually motile; some strains form desiccation-resistant cysts. C_1 compounds are assimilated via the ribulose monophosphate pathway. They are neutrophilic, the pH range for growth spans from 5.5 to 9.0, with optimal growth at about pH 7.0. The majority of species are mesophilic, and most strains grow between 15 and 40 °C, with optimal growth between 23 and 35 °C. Some representatives, like *M. tundripaludum* and *M. psychrophilus*, are psychrotolerant and psychrophilic. Two species require sodium ions for growth. None of the *Methylobacter* species has been reported to fix dinitrogen. These methanotrophs are typical inhabitants of freshwater and saline lake sediments, river and wetland muds, activated sludge, arctic and tundra soils, wastewater, and seawater (Kalyuzhnaya 2017).

Members of the genus *Methylomicrobium* possess rod-shaped, motile cells, which form regular glycoprotein S-layers arranged in *p2*, *p4*, or *p6* symmetries (Kalyuzhnaya 2016a). Cysts or other resting bodies are not formed. These aerobic methanotrophs can also grow at low oxygen tension and display fermentation and denitrification capabilities. They assimilate formaldehyde via the ribulose monophosphate pathway, and all strains have a partial serine cycle. Most members of this genus are mesophiles, with optimal growth at 25–35 °C. Some representatives are alkalitolerant or alkaliphilic, growing well in the pH range between 9 and 10.5, and require sodium ions for growth. These methanotrophs inhabit sediments of freshwater lakes and rivers, saline soda lakes, wetland muds, agricultural and swampy soils, upper mixing layers of oceans, and estuarine waters.

The genus *Methylosarcina* is represented by pleomorphic cells, which tend to grow in irregularly shaped sarcinal packets or aggregates (Kalyuzhnaya 2016b). Some members of this genus produce extracellular fibrils and form an extensive fibrillar matrix. They are mesophilic and neutrophilic bacteria, although *M. fibrata* and *M. lacus* grow best in slightly acidic conditions (pH 5.5–6.5). Genomes include complete sets of genes essential for operation of the ribulose monophosphate pathways and the serine cycle for carbon assimilation. Soluble MMO is lacking in cells of these methanotrophs; they are also incapable of dinitrogen fixation. Habitats are various terrestrial ecosystems including landfill soils, freshwater sediments, rice paddies, and grassland soils.

Representatives of the genus *Methylocaldum* possess coccoidal to rod-shaped pleomorphic cells, produce cysts, and form light to dark brown-colored colonies. *Methylocaldum* species possess key enzymes for the ribulose monophosphate and the serine pathways of formaldehyde assimilation. These methanotrophs do not fix dinitrogen. All members of this genus are thermotolerant methanotrophs that grow at temperatures of up to 62 °C (*M. szegediense*) or 47 °C (other described species). None of the described *Methylocaldum* species can grow below 20 °C. These methanotrophs have been detected in diverse environments including marine and aquatic habitats, upland soils, rice fields, and landfills (Takeuchi 2016).

Methanotrophs of the genus *Methylogaea* are slightly curved, nonmotile cells with rounded ends. They are neutrophilic and mesophilic bacteria, which possess only pMMO and grow optimally at 30–35 °C. Although a *nifH* gene is present, tests for nitrogenase activity were negative. The type strain of the only currently described species of this genus has been isolated from a flooded rice field (Tarlera 2016).

The genus *Methyloparacoccus* also includes a single species, *M. murrellii*, which was isolated from pond water (Hoefman et al. 2014). It is characterized by nonmotile, coccoid cells that tend to occur in pairs and contain only pMMO. These methanotrophs are neutrophilic, mesophilic and incapable of dinitrogen fixation.

Members of the genera *Methylosoma* and *Methyloglobulus* are microaerobic methanotrophs that grow best at low oxygen tensions (Schink and Rahalkar 2016; Schink and Deutzmann 2016). Cells are nonmotile, short rods or cocci that occur in pairs or in short chains. These mesophilic and neutrophilic methanotrophs do not possess sMMO and are capable of dinitrogen fixation. They inhabit sediments of freshwater lakes and occur at the interface of oxic and anoxic methane-supplied sediment layers.

The genus *Methyloprofundus* contains a single species, *M. sedimenti*, which was isolated from surface sediments in the deep ocean. Cells of these methanotrophs are nonmotile elongated cocci that occur singly, in pairs, or in clumps; resting cells are not formed. They are mesophilic to psychrotolerant (growing down to 4 °C) and slightly halophilic and are capable of dinitrogen fixation. The ribulose monophosphate pathway is used to assimilate formaldehyde into cellular carbon. Members of this genus have been detected exclusively in the deep ocean, most typically in methane-rich seeps and sediments, and within bacteriocytes of seep-associated mussels in *Bathymodiolus* (Tavormina 2016).

Representatives of the genus *Methylomarinum* were also isolated from marine environments, but in contrast to *Methyloprofundus*, they colonize shallow submarine hydrothermal systems and coastal marine sediments. Cells are short rods or oval shaped and are motile by a single polar flagellum. No cysts are formed. These are mesophilic methanotrophs, which require NaCl (1–8%, w/v) for growth and do not fix dinitrogen. C_1 compounds are assimilated via the ribulose monophosphate pathway (Hirayama 2016a).

The only currently described representative of the genus *Methylomagnum* was isolated from the rice rhizosphere (Khalifa et al. 2015). Cells are motile rods that contain both pMMO and sMMO. These methanotrophs are mesophilic and neutrophilic. The ribulose monophosphate and/or ribulose bisphosphate pathways are used for carbon assimilation.

The genus *Methylosphaera* includes a single species, *M. hansonii*, which was isolated from an Antarctic meromictic lake of marine salinity (Bowman 2015b). Spherical cells of these methanotrophs contain gas vesicles and occur singly or in pairs. C_1 compounds are utilized via the ribulose monophosphate pathway; ribulose-1,5-bisphosphate carboxylase activity is not present. These methanotrophs are psychrophilic organisms, growing between −2 and 20 °C with an optimal temperature range of 10–15 °C. They are capable of dinitrogen fixation and require seawater salts for optimal growth.

According to the phylogenetic clustering shown in Fig. 2.1, the filamentous methanotroph *Crenothrix polyspora* also affiliates with the family *Methylococcaceae* and, therefore, is discussed in this section. This morphologically striking bacterium with a complex lifestyle was originally described by Ferdinand Cohn in 1870. It remained physiologically uncharacterized up to 2006 when Stoecker and coauthors reported its ability to oxidize methane (Stoecker et al. 2006). Though this bacterium has never been isolated in a pure culture, its original name as well as the name of the corresponding family (*Crenothrichaceae*) were included in the Approved Lists of Bacterial Names (Skerman et al. 1980). Both names, therefore, are validly published, and, formally, *Crenothrix polyspora* is assigned to the family *Crenothrichaceae*. The apparent need to reassign this bacterium to the family *Methylococcaceae* remains to be considered in the future. It should be noted, however, that the ability to grow on methane as the sole source of energy has never been demonstrated for *Crenothrix polyspora*. The *pmo* genes of this filamentous methanotroph are much more closely related to *amo* of recognized betaproteobacterial ammonia oxidizers than to the *pmo* of described methanotrophs (Fig. 2.2). Thus, many aspects of the physiology of *Crenothrix polyspora* remain to be elucidated. *Crenothrix*-like bacteria colonize drinking water wells, sewage treatment systems, and groundwater environments. They can also be found in rice paddies and water-saturated soils.

2.3.2 Family *Methylothermaceae*

The family *Methylothermaceae* was designated on the basis of 16S rRNA gene sequence phylogeny (Hirayama et al. 2014). At present, this family contains the genera *Methylothermus*, *Methylohalobius*, and *Methylomarinovum*. The family members are aerobic, neutrophilic methanotrophs that grow on methane and methanol, assimilate C_1 compounds via the ribulose monophosphate pathway, and are moderate thermophiles or slight/moderate halophiles (Hirayama 2016b). Cells contain an extensive intracytoplasmic membrane system common to gammaproteobacterial methanotrophs. Soluble MMO is lacking in cells of these bacteria; they are also incapable of dinitrogen fixation. Habitats are thermal and/or saline environments.

The genus *Methylothermus* is the type genus of this family. It is represented by coccoid, motile, or nonmotile cells, which use only methane or methanol (Hirayama 2016c). These methanotrophs are moderately thermophilic, growing at a range of 37–67 °C with an optimal temperature of 55–60 °C. Members of this genus do not require NaCl for growth. They are common inhabitants of terrestrial hot springs.

Members of the genus *Methylomarinovum* are less thermophilic than *Methylothermus* spp. (growth range 30–55 °C with optimum at 45–50 °C). Cells are motile cocci or oval-shaped short rods. These methanotrophs were isolated from marine environments and require NaCl for growth (optimal growth occurs at 3% NaCl) (Hirayama 2016d).

The genus *Methylohalobius* comprises moderately halophilic, mesophilic, obligately methanotrophic bacteria (Dunfield 2016). They have the highest salt tolerance

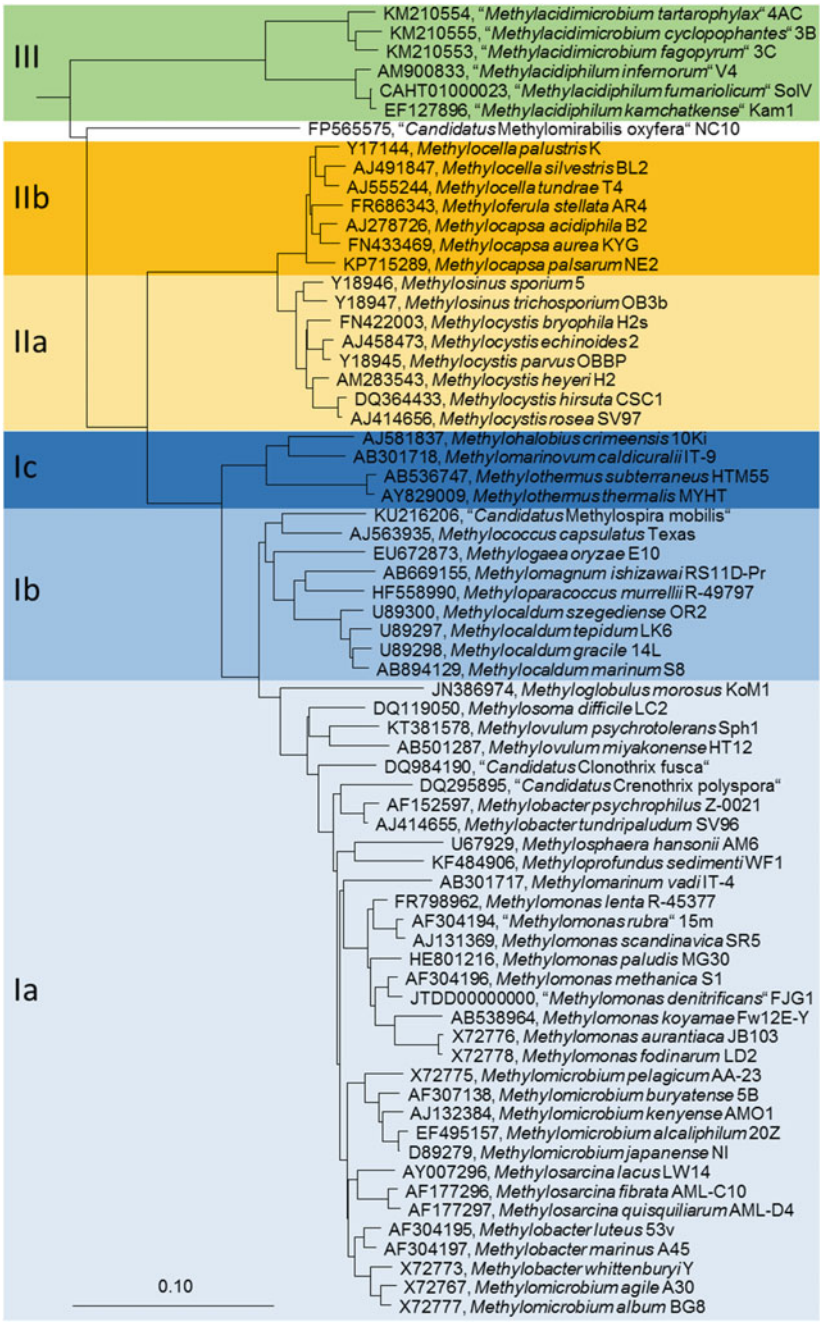

Fig. 2.1 Phylogeny of described aerobic methanotrophic bacteria based on 16S rRNA gene sequences. A neighbor-joining tree was calculated with Jukes Cantor correction based on 1556 nucleotide positions using the ARB software package (Ludwig et al. 2004). The tree was rooted with sequences of methanogenic *Archaea* (AB301476, M60880, AB065296, AM114193, AB196288). The scale bar displays 0.10 changes per nucleotide position

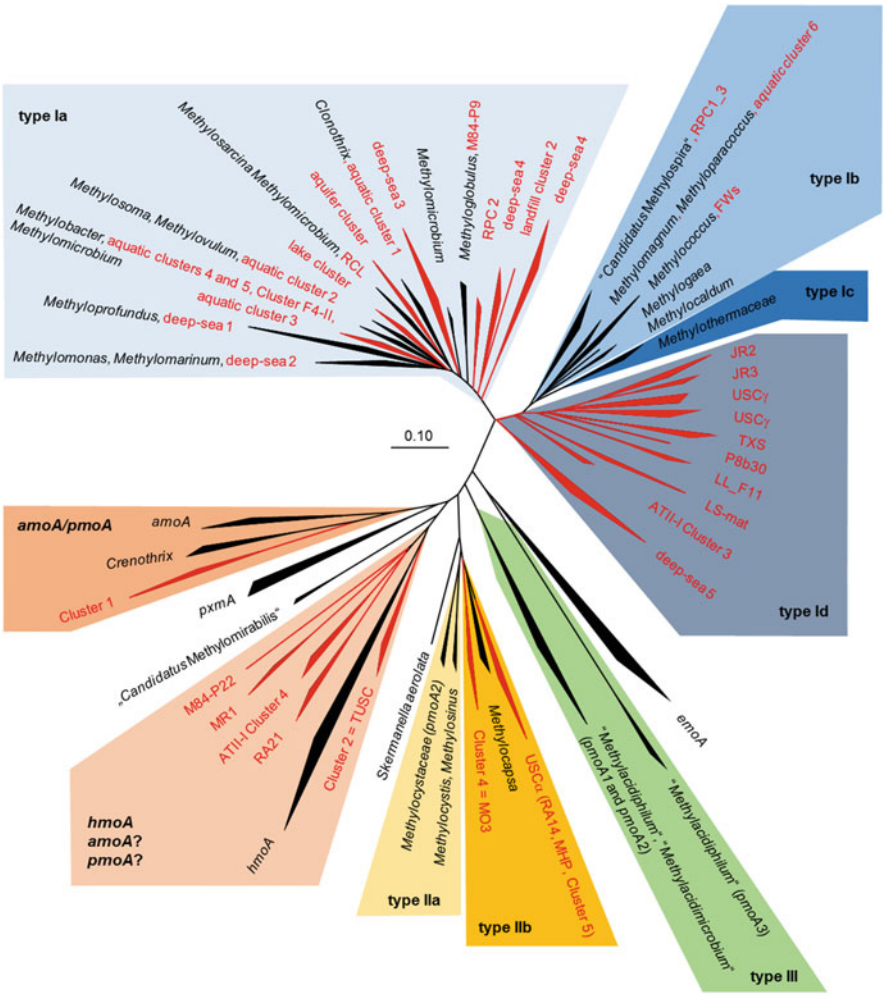

Fig. 2.2 Radial neighbor-joining tree showing the *pmoA*-based phylogeny of uncultivated clusters of methanotrophs (highlighted in red) in relation to cultivated representatives (displayed in black). The tree was calculated based on 480 nucleotide positions with Jukes Cantor correction. The scale bar displays 0.10 changes per nucleotide position. The grouping into clusters was done based on representative reference sequences. A distinct grouping of uncultivated groups and cultivated genera was not always possible, resulting in some mixed clusters

of any methanotroph yet cultured, growing optimally at 1–1.5 M NaCl and tolerating NaCl concentrations up to 2.5 M (14.6% w/v). Cells are nonpigmented, motile, coccoid, or spindle shaped and occur singly, in pairs, or in short chains. Known habitats are hypersaline lakes.

2.3.3 Family *Methylocystaceae*

This family belongs to the class *Alphaproteobacteria*, order *Rhizobiales*, and includes two genera of bacteria with methanotrophic capabilities, i.e., *Methylocystis* and *Methylosinus*. Cells of these methanotrophs contain type II intracytoplasmic membranes, which are arranged as layers in parallel to the periphery of the cell wall. C_1 compounds are assimilated via the serine pathway. Most members of this family are capable of fixing dinitrogen by means of an oxygen-sensitive nitrogenase.

Methylocystis is the type genus of this family. Cells are small, rodlike to reniform in shape and nonmotile. Resting cell forms are desiccation-resistant lipid cysts. Most representatives of this genus are obligate utilizers of one-carbon compounds, but several species are also capable of slow growth on acetate in the absence of methane. These methanotrophs grow in a wide range of temperatures (5–40 °C) and pH (4.5–9.0). *Methylocystis* species are among the ecologically most relevant methanotroph populations in terrestrial environments. They inhabit different soils, rice paddies, peatlands, landfills, and freshwater sediments (Belova et al. 2013; Bowman 2015c).

The genus *Methylosinus* is represented by pyriform or vibrioid-shaped cells, which are usually arranged in rosettes. They reproduce by binary and budding division. In budding division, the bud contains a heat and desiccation-resistant exospore, which germinates into a vegetative daughter cell; this daughter cell is motile. Members of this genus are obligately methanotrophic, mesophilic (optimal temperature 25–30 °C), neutrophilic (optimal pH 6.5–7.0), and non-halophilic. Major habitats include soil, freshwater sediments, and groundwater (Bowman 2015d).

2.3.4 Family *Beijerinckiaceae*

The family *Beijerinckiaceae* accommodates Gram-negative, aerobic, moderately acidophilic bacteria, which divide by binary or irregular fission. Members of this family display extremely versatile metabolic types including facultative and obligate methanotrophs, facultative methylotrophs, chemoheterotrophs, and anoxygenic phototrophs (Dedysh et al. 2016; Dedysh and Dunfield 2016a). Methanotrophic representatives of this family belong to the genera *Methylocapsa*, *Methylocella*, and *Methyloferula*. These methanotrophs were discovered in various acidic terrestrial environments such as *Sphagnum* peat bogs, tundra wetlands, and forest soils. All methanotrophs in this family are capable of dinitrogen fixation.

The genus *Methylocapsa* accommodates pMMO-possessing methanotrophic bacteria (Dedysh 2016). Cells contain a well-developed intracytoplasmic membrane system, which appears as stacks of membrane vesicles packed in parallel on only one side of the cell membrane. Some representatives of the genus *Methylocapsa* are obligate utilizers of one-carbon compounds, while others are also capable of growth on acetate, but sugars are not utilized. C_1 compounds are assimilated via the serine pathway.

Members of the genera *Methylocella* and *Methyloferula* are also methanotrophic bacteria, but in contrast to *Methylocapsa* species and all other known aerobic methanotrophs, they employ only a soluble methane monooxygenase (sMMO) for methane oxidation (Dedysh and Dunfield 2016b, c). Intracytoplasmic membranes are lacking from cells of these unusual methanotrophs. Although both are methanotrophic, *Methylocella* and *Methyloferula* differ with regard to their substrate utilization patterns. *Methylocella* species are facultative methanotrophs, which, in addition to C_1 compounds, can utilize acetate and several other organic acids, ethanol, and some short-chain alkanes as energy and carbon sources. By contrast, *Methyloferula* grows only on methane and methanol. C_1 compounds are utilized via the serine pathway in *Methylocella* species and via the serine and ribulose bisphosphate pathways in *Methyloferula*.

2.3.5 Methanotrophs of the Phylum *Verrucomicrobia*

The known diversity of aerobic methanotrophic bacteria was further expanded by the discovery of methanotrophic bacteria within the phylum *Verrucomicrobia* (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008). These extremely acidophilic and thermophilic methanotrophs were assigned to the novel genus "*Methylacidiphilum*" (Op den Camp et al. 2009). Recently, a second genus within the newly formed methanotrophic family "*Methylacidiphilaceae*" was proposed, "*Methylacidimicrobium*," consisting of three species (van Teeseling et al. 2014). Members of these three species are mesophilic acidophiles. Methanotrophic verrucomicrobia use pMMO to catalyze the first step of methane oxidation; sMMO is absent. Unlike most proteobacterial methanotrophs, however, they grow as autotrophs, using only CO_2 as carbon source via the Calvin cycle. It should be noted that verrucomicrobial methanotrophs have been characterized in all details, but their names are not yet validly published. These bacteria appear to be restricted to acidic geothermal environments (Sharp et al. 2014).

2.3.6 Methanotrophic Bacteria with a *Candidatus* Status

In contrast to *Crenothrix polyspora*, the name of another filamentous bacterium, "*Candidatus* Clonothrix fusca," has not been validly published because, originally, it was considered a development stage of *C. polyspora*. De facto, however, both methanotrophs have never been isolated in pure cultures. One decade ago, "*Candidatus* Clonothrix fusca" was characterized as belonging to a novel genus of gammaproteobacterial methanotrophs distinct from *C. polyspora* (Vigliotta et al. 2007). "*Candidatus* Clonothrix fusca" possesses a conventional *pmoA* gene that is phylogenetically divergent from the unusual *pmoA* of *C. polyspora* (Fig. 2.2). Some slow growth with methanol or formaldehyde as the sole carbon source under laboratory conditions has also been demonstrated for "*Candidatus* Clonothrix

fusca." Both "*Candidatus* Clonothrix fusca" and *Crenothrix polyspora* are typically found in habitats with slowly running water, often attached to submerged surfaces.

"*Candidatus* Methylospira mobilis" is a recently described obligately microaerophilic methanotroph with spiral-shaped, motile cells (Danilova et al. 2016). The specific cell shape enables rapid motility of these bacteria in water-saturated, heterogeneous environments with high microbial biofilm content, therefore offering an advantage of fast cell positioning under desired high methane/low oxygen conditions. These methanotrophs were successfully cultivated in the laboratory with methane as the only growth substrate but, despite all purification efforts, could not be obtained in a pure culture. The satellite organisms were identified as *Magnetospirullum*- and *Methylobacterium*-like bacteria. The *pmoA* genes from "*Candidatus* Methylospira mobilis"-like organisms form a new genus-level lineage within the family *Methylococcaceae*, type Ib methanotrophs. These psychrotolerant and mildly acidophilic to neutrophilic methanotrophs inhabit northern freshwater habitats including peatlands, organic soils, and sediments.

2.4 Uncultivated Methanotrophic Bacteria

Cultivation-independent studies have revealed the existence of diverse groups of methanotrophs that have not yet been cultured. Most of these uncultivated methanotrophic bacteria are only known by their *pmoA* sequences, due to the fact that this gene, which encodes a subunit of the particulate methane monooxygenase, serves as molecular marker for methanotrophs in many studies. A comparison of publicly available *pmoA* sequences from uncultivated organisms and cultivated taxa revealed that approximately half of the *pmoA* sequences retrieved in cultivation-independent studies affiliate with described methanotrophic genera (Knief 2015). This shows that many type strains represent ecologically important populations of methanotrophs. Among the most frequently detected genera in cultivation-independent studies are in particular those that were already isolated in early studies, i.e., *Methylocystis*, *Methylosinus*, *Methylomonas*, *Methylobacter*, *Methylosarcina*, *Methylomicrobium*, *Methylococcus*, and *Methylocaldum.* These frequently detected taxa inhabit different ecosystems. In contrast, some other genera have only rarely been detected in environmental studies so far and appear to have a rather narrow ecological niche. These include *Methylomarinovum*, *Methylomarinum*, *Methylohalobius*, *Methyloglobulus*, or "*Methylacidimicrobium*."

Although a substantial proportion of the currently recognized methanotroph diversity is covered by cultured strains, phylogenetic trees based on *pmoA* sequences reveal a number of clusters without any cultivated representative (Fig. 2.2). Most of these clusters affiliate with the *Gammaproteobacteria*. They are found among type Ia, Ib, and Ic methanotrophs or form a distantly related branch, defined as type Id. Further clusters of uncultivated methanotrophs are related to type IIb (*Methylocapsa*) or are only distantly related to *pmoA* sequences of known methanotrophs. It remains unclear whether these highly divergent clusters represent

methanotrophic bacteria or organisms that harbor a monooxygenase with a different substrate preference, e.g., short-chain hydrocarbons or ammonium.

Several clusters of uncultivated methanotrophs have been termed after the ecosystem in which they are most commonly detected. This applies in particular to the deep-sea clusters 1–5, which are almost exclusively found in marine ecosystems (Lüke and Frenzel 2011). Notably, deep-sea clusters 1 and 2 have meanwhile cultivated representatives (*Methylomarinum* and *Methyloprofundus*, respectively), but the nomenclature of the clusters is kept due to the fact that the diversity within these clusters is likely to be higher than reflected by one genus. Together with deep-sea cluster 3, they represent type Ia methanotrophs. The phylogenetic placement of clusters 4 and 5 varies depending on the treeing method, so that the assignment of these groups to types Ia and Id has to be considered with care (Lüke and Frenzel 2011; Knief 2015). A bit less ecosystem specific but nevertheless characteristic for aquatic environments are the sequences of the aquatic clusters 1–6, the lake cluster, and cluster FWs. Aquatic cluster 1 is related to *Clonothrix*, while the exact positioning of the aquatic clusters 2, 4, and 5 is more variable depending on the treeing method and sequence data input. They are most closely related to *Methylosoma*, *Methylovulum*, and *Methylobacter*. Closely related to these is also aquatic cluster 3. Aquatic cluster 6 represents type Ib methanotrophs, being most closely related to *Methyloparacoccus*. Likewise, the lake cluster and cluster FWs represent type Ia and Ib methanotrophs, respectively.

Some terrestrial habitats also harbor specific groups of uncultivated methanotrophs, especially rice paddies and upland soils. Frequently detected in rice paddies are the rice paddy clusters RPC1, 2, and 3 (Lüke et al. 2010). RPC1 and RPC3 are sometimes combined into a larger cluster (RPC1_3-like) because they are closely related and cannot always be easily delineated (Knief 2015). They represent type Ib methanotrophs, while RPC2 shows variable clustering either with type Ia or Ib (Lüke and Frenzel 2011). It should be noted that the larger RPC1_3-like cluster includes some subgroups that are not typical for rice paddies (Knief 2015), as well as the recently described "*Candidatus* Methylospira mobilis" (Danilova et al. 2016). Habitat specificity is most evident for specific subclusters within this large clade. In particular RPC1 and cluster JRC3 were initially exclusively detected in rice paddy associated habitats (Lüke et al. 2010; Lüke and Frenzel 2011). Characteristic for several upland soils is the presence of upland soil clusters α (USCα) and USCγ. While USCα represents type IIb methanotrophs, USCγ is member of the type Id methanotrophs. Both groups cover a broad diversity of sequences and can be divided into different subclusters (Shrestha et al. 2012; Knief 2015). They are assumed to represent organisms involved in atmospheric methane oxidation (Dunfield 2007; Kolb 2009). Moreover, cluster 2 (or tropical upland soil cluster = TUSC) is typical for upland soils, but as this cluster is related to hydrocarbon monooxygenase genes, it remains unknown whether it represents methanotrophic bacteria (Knief 2015).

Several further clusters of *pmoA* sequences have been defined in the literature and are shown in Fig. 2.2. Their detection is mostly not limited to a specific habitat so

that their ecological niche cannot yet be defined. They are mostly named after the name of a representative clone.

2.5 Final Remarks

As outlined above, the large proportion of the currently recognized diversity of aerobic methanotrophic bacteria is now brought into culture, although several *pmoA* lineages still lack cultivated members and represent the challenge for further cultivation studies. It should also be taken into account that some methanotrophs may possess only a soluble MMO and, therefore, cannot be detected using a *pmoA*-based PCR assay considered universal and specific for all other known methanotrophs. By recently, the number of these "unusual," pMMO-lacking methanotrophs was limited by members of the genera *Methylocella* and *Methyloferula*. In 2016, however, one additional methanotroph with similar characteristics was described (Vekeman et al. 2016). This sMMO-containing marine microorganism is a member of the earlier described genus *Methyloceanibacter* that accommodates methylotrophic bacteria incapable of growth on methane. This discovery suggests the possibility of as-yet-unexplored metabolic and physiologic variability within certain described taxa of methylotrophs as well as within uncultivated groups of these bacteria.

Future studies will be greatly facilitated by the availability of complete genomic information, which has been obtained for the majority of described methanotroph genera in the meantime (Table 2.2). Most often, only the type strains have been sequenced. In some cases, however, genomic information is available for one or more additional strains within a genus. This applies in particular to *Methylobacter*, *Methylomonas*, *Methylocystis*, and *Methylosinus*. Most genome sequences have been released as drafts, i.e., they consist of several scaffolds. Completely closed genomes are primarily available for the early sequenced strains of *Methylococcus capsulatus*, *Methylomicrobium album*, *Methylomicrobium alcaliphilum*, *Methylomonas methanica*, *Methylocystis* sp., *Methylocella silvestris*, or two of the "*Methylacidiphilum*" strains. Increasing the number of high-quality genome sequences from both cultivated and as-yet-uncultivated methanotrophs should open the way to a genome-based taxonomy of these bacteria.

Table 2.2 Genome sequenced strains of aerobic methanotrophic bacteria, compiled based on the publication of data in the public NCBI database

Genus strain	Sequence accession	Assembly level [scaffolds (contigs)][a]	Reference
Methylobacter			
M. luteus IMV-B-3098	ATYJ00000000	4 (17)	Hamilton et al. (2015)
M. marinus A45^T	ARVS00000000	2 (12)	Flynn et al. (2016)
M. tundripaludum SV96^T	AEGW00000000	3 (17)	Svenning et al. (2011)
M. tundripaludum 21/22	JMLA00000000	1	Kalyuzhnaya et al. (2015)
M. tundripaludum 31/32	JPOH00000000	2	Kalyuzhnaya et al. (2015)
M. whittenburyi UCM-B-3033	JQNS00000000	7	Hamilton et al. (2015)
Methylobacter sp. BBA5.1	JQKS00000000	88 (92)	Flynn et al. (2016)
Methyloglobulus			
M. morosus KoM1^T	AYLO00000000	183	Poehlein et al. (2013)
Methylomarinum			
M. vadi IT-4^T	JPON00000000	1	Flynn et al. (2016)
Methylomicrobium			
M. agile ATCC35068^T	JPOJ00000000	4	Hamilton et al. (2015)
M. album BG8^T	CM001475 CM001476 (plasmid)	1 (2) 1	Kits et al. (2013)
M. alcaliphilum 20Z^T	FO082060 FO082061 (plasmid)	1 1	Vuilleumier et al. (2012)
M. buryatense 5G^T	AOTL00000000	2 (26)	Khmelenina et al. (2013)
Methylomonas			
"*M. denitrificans*" FJG1^T	CP014476	1	Kits et al. (2015)
M. koyamae JCM 16701^T	BBCK00000000	283	
M. koyamae R-45378	LUUJ00000000	145	Heylen et al. (2016)
M. koyamae R-45383	LUUK00000000	235	Heylen et al. (2016)
M. koyamae R-49807	LUUL00000000	147	Heylen et al. (2016)
M. lenta R-45370	LUUI00000000	171	Heylen et al. (2016)
M. methanica MC09	CP002738	1	Boden et al. (2011)
M. methanica S1^T	LUUF00000000	115	Heylen et al. (2016)
M. methanica R-45363	LUUG00000000	139	Heylen et al. (2016)
M. methanica R-45371	LUUH00000000	120	Heylen et al. (2016)
Methylomonas sp. DH-1	CP014360 CP014361 (plasmid)	1 1	

(continued)

Table 2.2 (continued)

Genus strain	Sequence accession	Assembly level [scaffolds (contigs)][a]	Reference
Methylomonas sp. LWB	MKMC00000000	100 (105)	
Methylomonas sp. 11b	AZXK00000000	1 (2)	Kalyuzhnaya et al. (2015)
Methylomonas sp. LW13	JNLB00000000	42	Kalyuzhnaya et al. (2015)
Methylomonas sp. MK1	AQOV00000000	5	Kalyuzhnaya et al. (2015)
Methylosarcina			
M. fibrata AML-C10^T	ARCU00000000	2 (6)	Hamilton et al. (2015)
M. lacus LW14^T	AZUN00000000	1	Kalyuzhnaya et al. (2015)
Methylovulum			
M. miyakonense HT12^T	AQZU00000000	1 (9)	
Methylocaldum			
M. szegediense O-12	ATXX00000000	2 (108)	
Methylococcus			
M. capsulatus Texas^T	AMCE00000000	114	Kleiveland et al. (2012)
M. capsulatus Texas^T	AUKJ00000000	20 (24)	
M. capsulatus Bath	AE017282	1	Ward et al. (2004)
Methylogaea			
M. oryzae JCM 16910^T	BBDL00000000	990	
Unclassified *Methylococcales*			
Methylococcaceae bacterium 73a	JYNS00000000	73 (74)	
Methylococcaceae bacterium Sn10-6	LAJX00000000	389	Rahalkar et al. (2016)
Methylococcales bacterium OPU3_GD_OMZ	MPSY00000000	304 (336)	
Methylohalobius			
M. crimeensis 10Ki^T	ATXB00000000	5	Sharp et al. (2015)
Unclassified *Methylothermaceae*			
Methylothermaceae sp. B42 (Ga0078419)	LSNW00000000	39 (40)	Skennerton et al. (2015)
Methylocystis			
M. parvus OBBP^T	AJTV00000000	108	del Cerro et al. (2012)
M. rosea SV97^T	ARCT00000000	2 (4)	
Methylocystis sp. ATCC49242 / Rockwell	AEVM00000000	6 (7)	Stein et al. (2011)
Methylocystis sp. LW5	JMKQ00000000	6	
Methylocystis sp. SB2	AYNA00000000	149 (158)	Vorobev et al. (2014)

(continued)

Table 2.2 (continued)

Genus strain	Sequence accession	Assembly level [scaffolds (contigs)][a]	Reference
Methylocystis sp. SC2	HE956757 FO000001 (plasmid 1) FO000002 (plasmid 2)	1 1 1	Dam et al. (2012, 2013)
Methylosinus			
M. trichosporium OB3b ^{T}	ADVE00000000	3	Stein et al. (2010)
Methylosinus sp. 3S-1	LXWX00000000	159	Bao et al. (2016)
Methylosinus sp. LW3	AZUO00000000	5 (6)	
Methylosinus sp. LW4	ARAB00000000	3 (16)	
Methylosinus sp. PW1	JQNK00000000	12	
Methylosinus sp. R-45379	LUUM00000000	319	Heylen et al. (2016)
Methylocapsa			
M. acidiphila B2^{T}	ATYA00000000	2 (6)	Tamas et al. (2014)
M. aurea KYG^{T}	JQKO00000000	34 (37)	
M. palsarum NE2^{T}	FOSN00000000	78	
Methylocella			
M. silvestris BL2^{T}	CP001280	1	Chen et al. (2010)
Methyloferula			
M. stellata AR4^{T}	ARWA00000000	1	Dedysh et al. (2015)
Methylacidiphilum			
"*M. fumariolicum*" SolV^{T}	LM997411	1	Khadem et al. (2012), Anvar et al. (2014)
"*M. infernorum*" V4^{T}	CP000975	1	Hou et al. (2008)
"*M. kamchatkense*" Kam1^{T}	JQNX00000000	41	Erikstad Birkeland (2015)
Methylacidimicrobium			
"*M. fagopyrum*" 3C^{T}	ARAS00000000	8 (139)	van Teeseling et al. (2014)
"*M. cyclopophantes*" 3B^{T}	SRX752827	Unassembled data	van Teeseling et al. (2014)
Unclassified *Verrucomicrobia*			
Verrucomicrobia bacterium LP2A	JAFS00000000	3	Sharp et al. (2014)

[a]According to the data available from the NCBI genome database, number of contigs is given when higher than number of scaffolds

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Verrucomicrobial Methanotrophs

3

Huub J. M. Op den Camp, Sepehr S. Mohammadi, Arjan Pol, and Peter F. Dunfield

3.1 Methane and Methanotrophy

The well-studied methanotrophic members of the Alpha- and Gammaproteobacteria have never been found in methane rich environments of extremely low pH. However, methane oxidation activity was detected in geothermal soils characterized by high temperatures (50–95 °C) and a pH as low as 1.0. From 2007 onward, the isolation of new aerobic acidophilic methane oxidizing bacteria from several acidic geothermal ecosystems was reported. They were all identified as new members of the Verrucomicrobia phylum and clustered in the genera *Methylacidiphilum* (thermophiles) and *Methylacidimicrobium* (mesophiles). This chapter reports on their discovery, biodiversity, genomics, metabolism, and biotechnological potential.

Methane is released to the atmosphere from natural and anthropogenic sources. The most important sources include natural ecosystems (e.g., wetlands, ruminants, and termites) and anthropogenic activities (e.g., rice paddy fields, landfills, and mining). In these systems, microbial biogenesis of methane is carried out by methanogenic Archaea during organic matter decay under anoxic conditions (Thauer 1998; Conrad 2009; Etiope et al. 2011). In addition, non-microbial methane is emitted to the atmosphere from geothermal regions like cold seeps, mud volcanoes, and fumaroles. This methane is produced primarily via the thermal decomposition of organic matter (>80 °C) in the earth's crust (Etiope and Klusman 2002; Conrad 2009; Etiope et al. 2011). Besides emission sources, sinks of methane are also present on our planet. Atmospheric methane can react with the hydroxyl radical $OH^{\cdot}$ leading to the formation of carbon dioxide and water vapor (Jacob 1999). Methane diffusing from anoxic production zones toward the atmosphere can be

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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_3

oxidized by both aerobic and anaerobic methane-oxidizing microorganisms, also known as methanotrophs (Forster et al. 2007; Conrad 2009). These microorganisms are assumed to be the major players in keeping the methane balance on our planet by oxidizing 50–80% of all methane produced before it can reach the atmosphere (Moss et al. 2000; Forster et al. 2007; Conrad 2009).

Microbial oxidation of methane can occur with or without molecular oxygen (O_2). To date we know that anaerobic oxidation of methane (AOM) can utilize various terminal electron acceptors: sulfate (Knittel and Boetius 2009; Milucka et al. 2012), nitrite (Ettwig et al. 2008), nitrate (Raghoebarsing et al. 2006; Haroon et al. 2013; Arshad et al. 2015), and manganese and iron oxides (Beal et al. 2009; Egger et al. 2015; Scheller et al. 2016). Many other microorganisms can oxidize methane aerobically, with O_2 as the terminal electron acceptor. For many years it was believed that aerobic methanotrophy was a phenotype only present in a few bacteria belonging to the phylum Proteobacteria, specifically in the classes Alphaproteobacteria (also named type II methanotrophs) and Gammaproteobacteria (type I methanotrophs) (Hanson and Hanson 1996; Dedysh et al. 2000; Op den Camp et al. 2009). The two groups differ in their pathways for biomass production, the ribulose monophosphate (RuMP) pathway in the Gammaproteobacteria versus the serine pathway in the Alphaproteobacteria (Hanson and Hanson 1996; Chistoserdova et al. 2009; Chistoserdova 2011). Other broad physiological and structural characteristics separating the two groups (cell shape, ultrastructure of intracytoplasmic membranes, the main phospholipid fatty acids, nitrogen fixation ability, presence of different monooxygenases, etc.) were also defined in the early years of research into these bacteria (Hanson and Hanson 1996), but recently many genera have been discovered that do not fit these sweeping generalizations (e.g., *Methylocella*, *Methylohalobius*, *Methylovulum*, and *Methyloceanibacter*; Dedysh et al. 2000; Heyer et al. 2005; Iguchi et al. 2011; Vekeman et al. 2016).

3.2 The Discovery of the Aerobic Verrucomicrobial Methanotrophs

The methanotrophic members of the Alpha- and Gammaproteobacteria have never been found in methane-rich environments of extremely low pH (Op den Camp et al. 2009). The most acidophilic proteobacterial methanotrophs known, species of *Methylocella* and *Methylocapsa*, grow in peat environments at pHs as low as 4.2 (Dedysh et al. 1998, 2002; Dunfield and Dedysh 2010). However, methane oxidation activity was detected in geothermal soils in the Solfatara at Pozzuoli near Naples (Italy), a site characterized by high temperatures (50–95 °C) and a pH as low as 1.0 (Castaldi and Tedesco 2005). The hydrogen sulfide present in the hot fumarolic gas at this site is oxidized into sulfuric acid biotically or abiotically, thereby forming an extremely acidic ecosystem. Biogeochemical evidence for methane oxidation at extremely low pH was confirmed in late 2007 to early 2008 by three independent studies that obtained isolates of new aerobic methane-oxidizing bacteria from several acidic volcanic samples from the Solfatara; from Hell's Gate, Tikitere (New Zealand); and from the Uzon Caldera, Kamchatka (Russia) (Pol et al. 2007;

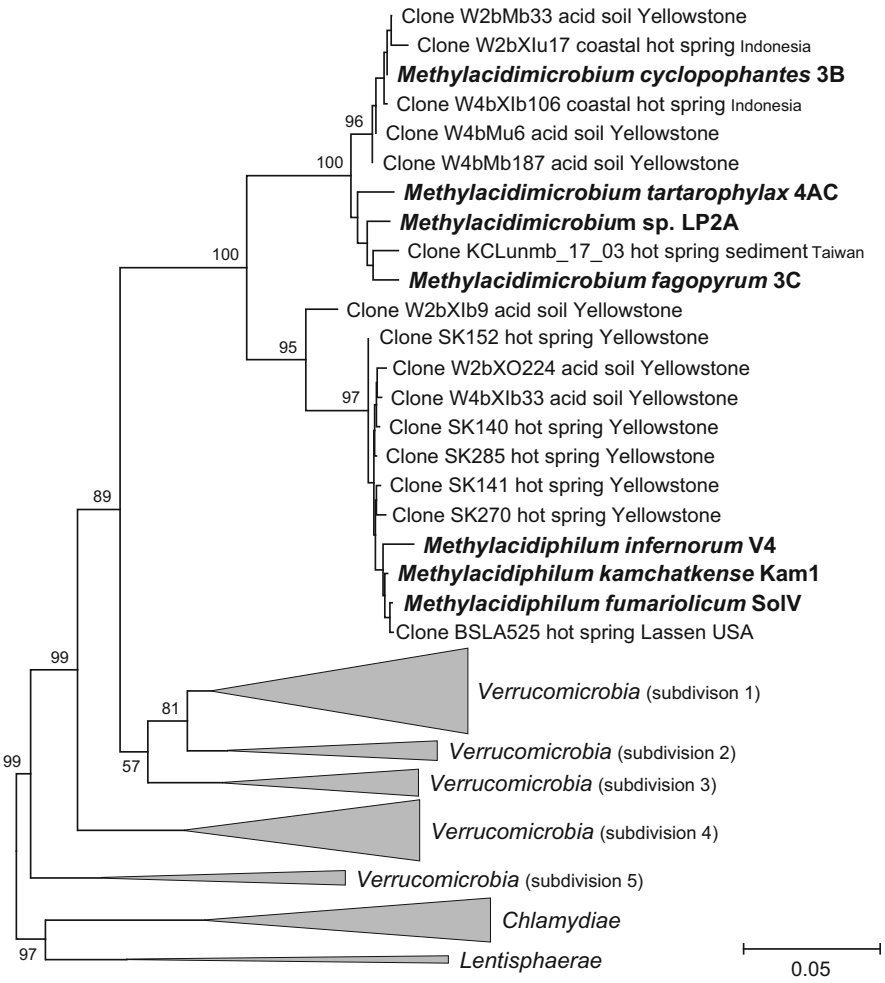

Fig. 3.1 Phylogenetic tree of 16S rRNA gene sequences of methanotrophic and other Verrucomicrobia showing the evolutionary relationships of the methanotrophic mesoacidophilic *Methylacidimicrobium* strains (3B, 3C, LP2A, and 4AC), the methanotrophic thermoacidophilic *Methylacidiphilum* strains (V4, SolV, and Kam1), and other members of the phylum *Verrucomicrobia* and other selected phyla. Cultivated representatives are indicated in boldface. Chlamydiae and Lentisphaerae species were used as outgroup

Dunfield et al. 2007; Islam et al. 2008). All three isolates were able to grow at pH 1 and temperatures up to 65 °C (Dunfield et al. 2007; Pol et al. 2007; Islam et al. 2008; Op den Camp et al. 2009). Interestingly, based on 16S ribosomal RNA gene analysis, all three strains (SolV, V4, and Kam1) were identified as members of the Verrucomicrobia phylum and phylogenetically formed a single genus-level cluster for which the name *Methylacidiphilum* was suggested (Fig. 3.1) (Op den Camp et al. 2009). This genus has yet to be taxonomically validated because of difficulties in maintaining a viable culture in reference culture collections, but is a useful label for

consistent identification. The link between a member of the widely distributed Verrucomicrobia phylum and a major global process like the methane cycle was an exciting discovery. Despite the importance of Verrucomicrobia in many different ecosystems, which is suggested by their abundance in 16S rRNA amplicons from environmental DNA extracts, the portion of cultivated representatives within the Verrucomicrobia is quite low (Hugenholtz et al. 1998). 16S rRNA gene phylogenetic analyses have allowed a division of the phylum into five subdivisions (Fig. 3.1), but the physiology of the different subdivisions is still poorly understood (Wagner and Horn 2006).

3.3 Diversity of Verrucomicrobia methanotrophs

After the discovery of the thermoacidophilic genus *Methylacidiphilum*, van Teeseling et al. (2014) and Sharp et al. (2014) isolated, characterized, and described four new species of mesophilic acidophilic verrucomicrobial methanotrophs from low-temperature geothermal sites: the soil of the Solfatara crater, which is at the center of the Campi Flegrei caldera, near Naples (Italy), and a geothermally impacted soil in Reporoa, New Zealand. The 16S rRNA genes of these new isolates were very similar to each other, but were less than 90% identical to those of the *Methylacidiphilum* species described earlier. Furthermore, Pmo*A*- and MxaF-/*XoxF*-based phylogenies showed similar clustering compared to the 16S rRNA-based phylogeny (Keltjens et al. 2014; van Teeseling et al. 2014). The new genus name *Methylacidimicrobium* was proposed for these mesophilic verrucomicrobial methanotrophs, including the species *Methylacidimicrobium tartarophylax* 4AC, *Methylacidimicrobium fagopyrum* 3C, *Methylacidimicrobium cyclopophantes* 3B, and *Methylacidimicrobium* sp. LP2A (van Teeseling et al. 2014). Again these names are not yet taxonomically validated.

Together, the thermophilic and mesophilic methanotrophs described form a coherent class-level clade within the Verrucomicrobia phylum (Fig. 3.1). 16S rRNA gene sequences belonging to this group have since been detected in other acidic geothermal habitats of Europe, Asia, and North America, indicating their widespread occurrence in such systems (Kozubal et al. 2012; Li et al. 2016; Gagliano et al. 2014). In an extensive survey of environments in Canada and New Zealand using high-throughput sequencing of 16S rRNA amplicons, Sharp et al. (2014) found putative methanotrophic Verrucomicrobia in samples covering a broad temperature range (22.5–81.6 °C), but only under acidic conditions (pH <5.0) and only in geothermal systems. They could not be detected in acidic non-geothermal methane-rich environments like fens and bogs, suggesting that factors other than pH alone also affect their distribution. However, similar 16S rRNA sequences have recently been detected in highly acidic microbially induced concrete corrosion zones of sulfide-impacted sewage pipes (Pagaling et al. 2014). This suggests that methanotrophic Verrucomicrobia are indeed present in some non-geothermal ecosystems, at least H_2S-rich ones. The factors controlling the ecological range of these bacteria are not yet completely understood, but may be related to their rare earth metal requirement (see below) in addition to their preference for highly acidic conditions.

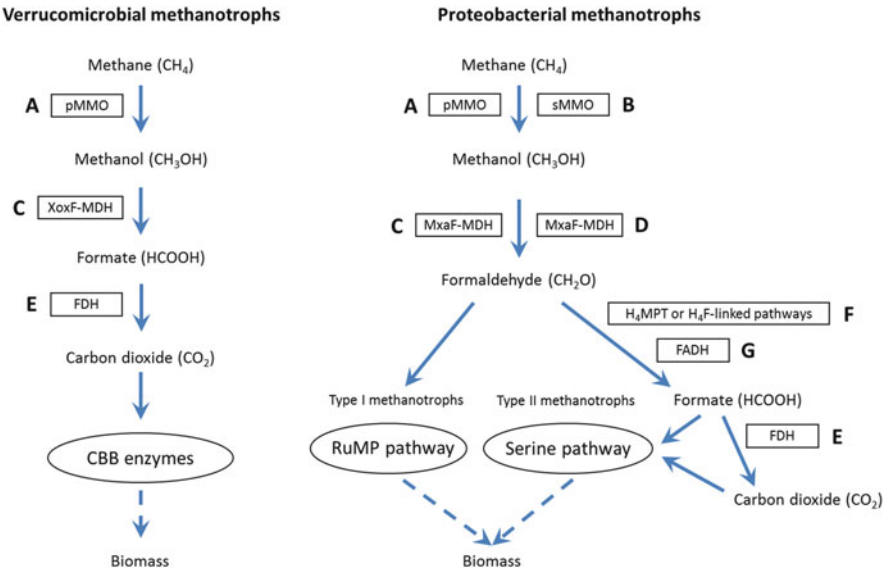

Fig. 3.2 Methane oxidation pathways in verrucomicrobial (left) and proteobacterial (right) methanotrophs with all enzymes (boxes) involved. (**A**) Particulate methane monooxygenase (pMMO); (**B**) soluble methane monooxygenase (sMMO). (**C**) Lanthanide-dependent methanol dehydrogenase (XoxF-MDH); (**D**) calcium-dependent methanol dehydrogenase (MxaF-MDH); (**E**) formate dehydrogenase (FDH); (**F**) multienzyme cofactor-linked C1 transfer pathways (H_4MPT = tetrahydromethanopterin, H_4F = tetrahydrofolate); (**G**) formaldehyde dehydrogenase (FADH). Formaldehyde is assimilated in type I proteobacterial methanotrophs via the ribulose monophosphate (RuMP), and formate is the main branch point to the serine pathways in type II proteobacterial methanotrophs. In verrucomicrobial methanotrophs, carbon dioxide is fixed using the Calvin-Benson-Bassham (CBB) cycle enzymes

3.4 Genomics and Metabolism of Verrucomicrobia Methanotrophs

The aerobic oxidation of methane has the net reaction, $CH_4 + 2O_2 \rightarrow CO_2 + 2H_2O$ ($\Delta G^\circ = -773$ KJ per mol CH_4), but proceeds microbially via the intermediates methanol (CH_3OH), formaldehyde (CH_2O), and formate (CHOOH) (Chistoserdova et al. 2009; Hanson and Hanson 1996) (Fig. 3.2). In this reaction scheme, electrons from methane are transported to a membrane-bound electron transport chain using a pyrroloquinoline quinone cofactor to cytochrome *c* (methanol dehydrogenase) or NAD (in formaldehyde oxidation systems and formate dehydrogenase). The terminal electron acceptor in aerobic methane oxidation is O_2.

The first step in methane oxidation is catalyzed by the methane monooxygenase enzyme (MMO) converting methane into methanol. This enzyme exists in two distinct forms; the soluble cytoplasmic form (sMMO, NADH-dependent) and the particulate membrane-associated form (pMMO, cytochrome *c* dependent) (Hanson and Hanson 1996; Fig. 3.2). In general, sMMO is located in the cytoplasm, and pMMO is situated in

the intracytoplasmic membranes (ICMs) present in many proteobacterial methanotrophs (Nguyen et al. 1998; Brantner et al. 2002). The soluble cytoplasmic methane monooxygenase (sMMO) is only present in a limited number of methanotrophs, usually in addition to pMMO (Chistoserdova 2011), but occasionally as the sole MMO (*Methylocella*, *Methyloferula*, and *Methyloceanibacter*) has only the sMMO enzyme (Dedysh et al. 2005; Dunfield and Dedysh 2010; Vekeman et al. 2016). The completed circular genomes of *M. infernorum* strain V4 and *M. fumariolicum* strain SolV lack the genes encoding for sMMO subunits (Pol et al. 2007; Hou et al. 2008; Op den Camp et al. 2009; Anvar et al. 2014). Draft genomes of *M. kamchatkense* (Kam1) and the mesophilic *Methylacidimicrobium* strains also lack sMMO-encoding genes (Erikstad and Birkeland 2015; Sharp et al. 2014; van Teeseling et al. 2014). On the other hand, complete *pmoCAB* operons encoding pMMO are found in the genome of every strain. The *Methylacidimicrobium* strains either contain a single *pmoCAB* operon (van Teeseling et al. 2014) or in the case of strain LP2A two nearly identical operons and an orphan third *pmoC* copy (Sharp et al. 2014). The completed thermoacidophilic *Methylacidiphilum* genomes each contain three complete *pmoCAB* operons and an orphan fourth *pmoC* copy (Op den Camp et al. 2009), while the draft genome of *M. kamchatkense* strain Kam1 showed three complete *pmoCAB* operons and a fourth operon without the *pmoB* gene (Op den Camp et al. 2009; Erikstad et al. 2012). Remarkably, the three paralogous operons in *Methylacidiphilum* are phylogenetically divergent from one another by up to 50% amino acid sequence. Alien Hunter, a program that uses nucleotide k-mer frequencies to identify potential lateral gene transfer (Vernikos and Parkhill 2006), predicts that the *pmoCAB*3 operon, the most divergent of the three, has been obtained by lateral gene transfer from another organism (Sharp et al. 2013). However, as no other organism is known with a similar operon, the source of this transfer is unknown.

This presence of diverse *pmo* copies in the *Methylacidiphilum* genomes is not unique, as some proteobacterial methanotrophs also possess multiple, divergent copies of *pmo* operons. A divergent *pmoCAB2* was reported to be present in many type II proteobacterial methanotrophs, including the model strain *Methylocystis* SC2 (Tchawa Yimga et al. 2003). Baani and Liesack (2008) reported that the enzymes encoded by the two paralogous operons in this model strain showed different apparent K_m values. Recently, a sequence-divergent *pmo* (named *pxm*) was also found in some type I proteobacterial methanotrophs (Tavormina et al. 2011). The existence of sequence-divergent copies may indicate different physiological functions under different environmental conditions. In *Methylacidiphilum* species, the functions of the three paralogous operons are not yet clear, although initial expression studies have been performed. Khadem et al. (2012a) showed that *pmoCAB1* and *pmoCAB2* operons of strain SolV are highly but differentially expressed under oxygen limitation and oxygen excess, respectively. Interestingly, the third *pmoCAB* operon (*pmoCAB3*) of strain SolV was not expressed under any of conditions tested (Khadem et al. 2012a). In *M. kamchatkense* strain Kam1, growth on methanol instead of methane resulted in a downregulation of all *pmoA* genes (Erikstad et al. 2012).

The second enzyme involved in the aerobic oxidation of methane is the PQQ-dependent methanol dehydrogenase (MDH) converting methanol into formaldehyde (Fig. 3.2). This enzyme includes a large and a small subunit encoded by *mxaFI* genes and needs pyrroloquinoline quinone as cofactor and a cytochrome *c* electron acceptor encoded by the *mxaG* gene. Remarkably, in all available verrucomicrobial aerobic methanotrophic strains (both *Methylacidiphilum* and *Methylacidimicrobium* spp.), the well-studied *mxaFJGIRSACKLDEHB* cluster encoding the canonical mxaF-type MDH was absent and substituted by *xoxFGJ*, while biogenesis of the cofactor pyrroloquinoline quinone is encoded by *pqqABCDEF* (Hou et al. 2008; Op den Camp et al. 2009; Pol et al. 2014; Keltjens et al. 2014; van Teeseling et al. 2014; Sharp et al. 2014). After the observation that growth of *M. fumariolicum* SolV was strictly depended on the addition of mudpot water from its natural environment, it was discovered that the purified XoxG-type MDH of *M. fumariolicum* strain SolV contains lanthanides at the catalytic site, while previously studied mxaF-type MDHs have calcium as active metal at this site (Pol et al. 2014; Keltjens et al. 2014). It was also shown that the growth of strain SolV is dependent on lanthanides at submicromolar concentrations, which could replace the mudpot water.

The presence of a lanthanide in the PQQ catalytic center seems to make XoxF-type MDHs more efficient catalysts in methanol conversion, since they efficiently oxidize not only methanol but also formaldehyde. The oxidation of methanol into formate may have major implications for the diversity of methylo- and methanotrophic catabolism and anabolism. The field of methano- and methylotrophy is a rapidly expanding puzzle of redundant anabolic and catabolic possibilities and opportunities (Chistoserdova et al. 2009; Chistoserdova 2011).

The third step in the proteobacterial aerobic oxidation of methane is the conversion of formaldehyde to formate. Formaldehyde is a toxic intermediate and must be maintained at nontoxic levels (Chistoserdova 2011). Different formaldehyde-oxidizing systems are present in methylotrophs (Chistoserdova 2011). Formaldehyde oxidation can be carried out by a formaldehyde dehydrogenase (FADH; Fig. 3.2). This single enzyme is linked to NAD or mycothiol (Chistoserdova 2011). In addition, formaldehyde oxidation can be performed by multienzyme cofactor-linked C1 transfer pathways. Two important pathways for formaldehyde oxidation in methylotrophs require H_4MPT (tetrahydromethanopterin) or H_4F (tetrahydrofolate) as cofactors (Chistoserdova et al. 2009; Chistoserdova 2011). The H_4MPT pathway is common in proteobacterial methanotrophs but appears to be missing from the genomes of all the methanotrophic Verrucomicrobia. Some parts of a H_4F pathway are present, but this pathway has not been verified. *M. fumariolicum* strain SolV lacks FADH as well as H_4MPT pathway-encoding genes, but the lanthanide-dependent XoxF-type MDH was shown to oxidize methanol directly to formate (see above), so a specialized formaldehyde oxidation system may not be needed by these methanotrophs.

The last step in methane oxidation is the conversion of formate into carbon dioxide by formate dehydrogenase (FDH; Fig. 3.2). The complete genome of strains V4 and SolV and the draft genome of strain Kam1 show that formate oxidation is

probably conducted by a NAD-dependent formate dehydrogenase and a membrane-bound formate dehydrogenase (Pol et al. 2007; Hou et al. 2008; Anvar et al. 2014; Erikstad and Birkeland 2015). In proteobacterial methanotrophs, formaldehyde and/or formate is assimilated via two main pathways: the *RuMP* and serine pathways which are important for Gammaproteobacteria (type I) and Alphaproteobacteria (type II) methanotrophs, respectively (Hanson and Hanson 1996; Chistoserdova et al. 2009). In the RuMP pathway, the unique enzymes hexulose-6-phosphate synthase and hexulose-6-phosphate isomerase catalyze the reactions to assimilate formaldehyde to form glyceraldehyde-3-phosphate as an intermediate (Hanson and Hanson 1996). In the serine pathway, the exclusive reactions are catalyzed by serine hydroxymethyltransferase (STHM), hydroxypyruvate reductase (HPR), malate thiokinase (MTK), and malyl coenzyme A lyase (MCL) (Hanson and Hanson 1996). In this pathway, both formate and carbon dioxide are utilized to produce acetyl coenzyme A for biosynthesis (Crowther et al. 2008; Šmejkalová et al. 2010). Based on the full genomes of the verrucomicrobial methanotrophs strain SolV and V4, and the transcriptome data of strain SolV, it is clear that these bacteria do not follow either the RuMP or the serine pathway (Hou et al. 2008; Op den Camp et al. 2009; Anvar et al. 2014). Rather, the verrucomicrobial methanotrophs are autotrophs, fixing carbon from carbon dioxide using the Calvin-Benson-Bassham (CBB) cycle (Khadem et al. 2011; Sharp et al. 2012). Recently, Rasigraf et al. (2014) also showed autotrophic carbon dioxide fixation via the Calvin-Benson-Bassham cycle by the denitrifying methanotroph *Methylomirabilis oxyfera* belonging to the NC10 phylum.

In addition to methane oxidation, *M. fumariolicum* strain SolV is able to fix N_2 at low oxygen concentrations using an extremely oxygen-sensitive nitrogenase (Khadem et al. 2010). Furthermore, the storage of carbon in strain SolV was demonstrated. Glycogen was formed in the cells once ammonium was depleted and methane still present (Khadem et al. 2012b). Gammaproteobacterial methanotrophs may also produce glycogen, while alphaproteobacterial methanotrophs have been shown to produce PHB (polyhydroxybutyric acid) as a storage compound (Linton and Cripps 1978; Pieja et al. 2011a, b; Eshinimaev et al. 2002).

3.5 Knallgas Bacteria

Recently, it was shown that the methanotroph *Methylacidiphilum fumariolicum* SolV could also grow as a real "Knallgas" bacterium consuming hydrogen/carbon dioxide in the absence of methane (Mohammadi et al. 2017). Two sets of hydrogen uptake hydrogenase genes were identified in the genome of strain SolV, encoding an oxygen-insensitive (*hhy*-type) and an oxygen-sensitive (*hup*-type) enzyme. Transcriptome analysis revealed that the *hhy*-type hydrogenase was constitutively expressed and active. This hydrogenase was supposed to support growth on hydrogen only at oxygen concentrations below 1.5%. When oxygen was further educed to

below 0.2%, expression of the oxygen-sensitive *hup*-type hydrogenase was induced, and the growth rate increased to about 60% of the rate on methane.

In an independent study, Carere et al. (2017) showed that the environmental isolate *Methylacidiphilum* sp. RTK17.1 sustained aerobic respiration and carbon fixation using methane and hydrogen as electron donors either in concert or separately depending on substrate availability. This lifestyle may have facilitated expansion of their niche space in geothermally influenced surface soils.

Taken together, these results suggest that in their natural environments, where both hydrogen and methane might be limiting, verrucomicrobial methanotrophs may operate primarily as "Knallgas" bacteria or prefer a mixotrophic lifestyle. In view of these findings, the role of hydrogen in methanotrophic ecosystems has to be revised, especially in soil, and related to consumption of atmospheric methane.

3.6 Biotechnology

The low cost of natural gas and biogas compared to other fuels makes them promising feedstocks for bioconversions. Possible bioproducts include polymers, single-cell protein, vitamins, carotenoids, compatible solutes, lipids, or methanol (Strong et al. 2015). As of yet, there are no published reports of the use of verrucomicrobial methanotrophs for bioconversion or indeed for any other biotechnological use. At the moment one can only speculate that their unique biochemistry compared to all other methanotrophs may present unique possibilities for bioproduct discovery and production.

Ecologically, the Verrucomicrobia methanotrophs occupy a niche separate from proteobacterial methanotrophs and may therefore also present unique possibilities for environmental biotechnology. For example, biofiltration of methane is a useful way of reducing the carbon footprint of waste gas streams that are economically not feasible to recover because they are intermittent, remote, and contaminated or have low flow rates or low methane contents (Hettiarachchi and Hettiaratchi 2011). Via biofiltration, the potent greenhouse gas methane is oxidized to CO_2, a gas with a global warming potential 34 times less than methane. The presence of H_2S in biogas or natural gas ("sour gas") is a general problem for the sustained operation of a methane biofilter and in some situations is also problematic for the economic recovery of fuel methane. The Verrucomicrobia methanotrophs are naturally adapted to sour gas sources and may therefore be useful in biofiltration of sour gas. Their unique tolerance may even make sour gas streams viable feedstocks for biotechnology without a desulfurization step. The observed co-occurrence of sulfur- and methane-oxidizing acidophiles in soured pipes (Pagaling et al. 2014) indicates the possibility for simultaneous H_2S and CH_4 removal from waste gas.

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Proteobacterial Methanotrophs, Methylotrophs, and Nitrogen

4

Lisa Y. Stein

4.1 Introduction

Research on intersections between single-carbon and nitrogen metabolism have revealed a number of unexpected insights that have expanded our view of how proteobacterial methanotrophs and methylotrophs impact biogeochemical cycles. Aside from assimilating nitrogen as an essential element for cellular growth and metabolism, methanotrophs and methylotrophs metabolize and transform a diversity of inorganic and organic nitrogenous molecules and release reactive nitrogen species as products. Thus, methanotrophs and methylotrophs play a major role in both the global carbon and nitrogen cycles. This chapter outlines the more recent discoveries and unusual nitrogenous molecules and pathways used by proteobacterial methanotrophs and methylotrophs for assimilation, respiration, and regulation of their activities. The role of nitrogen in axenic cultures, complex communities, and bioindustrial applications is discussed.

4.2 Nitrogen as an Essential Macronutrient

There are excellent reviews describing the basic physiology and metabolism of methanotrophs and methylotrophs that list the major nitrogen sources required to sustain growth of the various genera and species in culture as well as descriptions of assimilatory pathways that they utilize (Bowman 2006; Kelly et al. 2014; Webb et al. 2014). Interestingly, the standard medium for growth and maintenance of methanotrophic bacteria is nitrate mineral salts due to the long-held assumption that ammonium is inhibitory to methane oxidation due to its competition with methane for methane monooxygenase enzymes (Hanson and Hanson 1996). Direct

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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_4

comparison of growth on nitrate versus ammonium as N-source across strains has shown that the extent of ammonium inhibition is highly variable and can actually promote growth of some strains over nitrate (Nyerges et al. 2010). Furthermore, nitrite produced by some strains from ammonia oxidation or nitrate reduction can inhibit growth, implying that the optimal nitrogen source is highly strain-dependent (Nyerges and Stein 2009). The cellular mechanisms that govern sensitivity or resistance of any given strain to ammonium or nitrite are not well characterized; however, the variations from strain-to-strain are significant and likely influence performance in axenic culture as well as niche preference in the environment. For example, as discussed below, the acquisition of hydroxylamine detoxification or denitrification genes is a mechanism that very likely confers tolerance of a strain to ammonium or nitrite, respectively.

Among the alphaproteobacterial methanotrophs and methylotrophs, nitrogen fixation genes are common, and experiments have shown growth of several diazotrophic strains in the absence of a fixed N-source [reviewed in (Bowman 2006; Kelly et al. 2014; Webb et al. 2014)]. Methylotrophic isolates associated with root tissues and nodules are generally diazotrophic, and these strains encode both nodulation (*nodA*) and nitrogen fixation (*nifH*) genes. Gammaproteobacterial methanotrophs in the *Methylosphaera* and *Methylococcus* genera (*M. capsulatus*, *M. thermophilus*) have been reported to fix dinitrogen, along with the *Alphaproteobacteria* in the *Methylosinus*, *Methylocapsa*, and *Methylocystis* genera (Bowman 2006; Dam et al. 2013; Webb et al. 2014; Dedysh et al. 2015). The verrucomicrobial methanotroph, *Ca. Methylacidiphilum fumariolicum* SolV, was also shown to grow in the absence of a fixed N-source (Khadem et al. 2010), substantiating that diazotrophy is a widespread trait among methanotrophs that is not linked to a narrow phylogenetic lineage, although a greater diversity of *Alphaproteobacteria* are diazotrophic compared to other lineages.

The ecological role of methanotrophs in association with rice roots and sphagnum peat moss has been intensely investigated due to enrichment of methanotroph populations, particularly under low-N conditions (Bao et al. 2014; Larmola et al. 2014; Vile et al. 2014; Ho et al. 2016; Kostka et al. 2016; Minamisawa et al. 2016). While most of the plant-associated methanotrophs encode and often express N-fixation genes, reports are mixed as to whether they are primarily supplying carbon or fixed N to the plant. There appears to be a complex interplay between methanotrophs and other genera of diazotrophic bacteria that provide both carbon and nitrogen in these symbiotic plant-microbe communities. Active N-fixation by methanotrophs has been detected via stable isotope probing experiments in ^{15}N-amended soils (Buckley et al. 2008) and via catalyzed reporter deposition-fluorescence in situ hybridization in rice tissues (Bao et al. 2014). Yet, the extent and regulation of diazotrophy in methanotrophs and methylotrophs in either symbiotic or free-living conditions remains an interesting, and largely open, research question.

Complex organics as nitrogen sources are less commonly used to grow and maintain methylotrophs; however, some methanotrophic *Methylococcus* and *Methylomonas* strains can use yeast extract, casamino acids, and amino acids as

nitrogen sources (Bowman 2006). Some methylotrophs such as *Methylobacterium* strain JS178 can use unusual molecules as nitrogen sources, such as 4-nitro-2,4-diazobutanal (Fournier et al. 2005). Several methanotrophic isolates have been reported to express a functional urease and grow on urea as a sole N-source (Bowman et al. 1993; Bowman 2006; Webb et al. 2014). However, the ability to hydrolyze urea appears to be strain-specific as some methanotrophs only encode partial urea cycles and often lack genes for a functional urease. Some methylotrophic bacteria have also been reported to hydrolyze urea (Greenwood et al. 1998; Doronina et al. 2000), although again, this activity is reported on a strain-by-strain basis similarly to the methanotrophs and is not universal.

Thiocyanate is commonly used as a N-source for isolation and maintenance of methylotrophic *Methylobacter* strains (Kelly et al. 2014) but has not been reported as a N-source for cultivation of methanotrophs. Similarly, utilization of methylamine as a carbon and nitrogen source is particularly common to methylotrophs (Kelly et al. 2014) but not as common in methanotrophs. Methylamine is an intriguing nutrient for methylotrophs as it can be utilized as both a carbon and a nitrogen source. A recent study showed that *Methylobacterium* strains encoding both the cytoplasmic and periplasmic pathways for methylamine utilization can differentially regulate these pathways to primarily metabolize methylamine as a carbon/energy source or under N-limiting conditions, as an N-source (Nayak et al. 2016).

4.3 Co-metabolism and Nitrification in Methanotrophs

Early studies on methanotrophic isolates suggested that ammonium should inhibit methane oxidation due to its competitive inhibition of methane monooxygenase enzymes (Bédard and Knowles 1989). Because of the common evolutionary origin of ammonia- and particulate methane monooxygenases (Tavormina et al. 2011), both enzymes share a similar substrate range. However, growth on either ammonia or methane as a sole substrate requires either the hydroxylamine ubiquinone redox module (HURM) for ammonia oxidation (Klotz and Stein 2008) or the C1 oxidation module, which includes methanol dehydrogenase, formaldehyde dehydrogenase, and formate dehydrogenase, for methane oxidation. The specialized nature of these downstream modules permits growth of a particular bacterium on either ammonia or methane, but thus far there is not an isolate that can grow on both substrates. The ammonia- and methane/methanol-oxidation pathways produce a highly toxic intermediate, hydroxylamine or formaldehyde, respectively, whose oxidation supports cellular processes or, in the case of formaldehyde, is assimilated. The discovery of hydroxylamine dehydrogenase genes in the genomes of many methanotrophs suggested acquisition of a hydroxylamine detoxification mechanism to cope with ammonia co-metabolism by methane monooxygenase (Stein and Klotz 2011). Indeed, the *haoAB* genes encoding hydroxylamine dehydrogenase are strongly upregulated in methanotrophs exposed to high ammonium or hydroxylamine concentrations and are not expressed during growth on nitrate (Campbell et al. 2011; Dam et al. 2014).

Interestingly, the HAO proteins in methanotrophs are similar to those characterized in the anammox bacterium, *Kuenenia stuttgartiensis*, in that they lack a specific tyrosine residue that is essential for forming an enzyme that can efficiently drive the complete oxidation of hydroxylamine to nitrite (Maalcke et al. 2014). Instead, these enzymes favor oxidation of hydroxylamine to nitric oxide (NO), which would likely be reduced to nitrous oxide (N_2O) by nitric oxide reductase or by abiotic reactions. The conundrum, then, is how ammonia is oxidized efficiently to nitrite, and not to N_2O, by methanotrophs and whether HAO enzymes are even required for converting hydroxylamine to nitrite (Nyerges and Stein 2009). Furthermore, *Methylocystis* sp. Rockwell, a species with high rates of ammonia oxidation but little capacity to emit N_2O, encodes *haoAB* but lacks nitric oxide reductase (Nyerges et al. 2010). Incidentally, this strain is exceptionally resistant to high concentrations of ammonium but intolerant to nitrite as would be predicted by its cohort of nitrogen metabolism genes (Stein et al. 2011).

Together, the available data suggest that strains expressing HAO are more capable of oxidizing ammonia to nitrite via hydroxylamine, whereas those not encoding HAO are poor nitrifiers and are not particularly tolerant to high concentrations of ammonium (Nyerges and Stein 2009; Nyerges et al. 2010; Campbell et al. 2011; Stein and Klotz 2011). Also, some strains, like *Methylomonas denitrificans* FJG1 (formerly *Methylomonas methanica*), cannot oxidize ammonia to hydroxylamine (or nitrite) at all even though the pMMO is still competitively inhibited by ammonia (Nyerges and Stein 2009). Therefore, the dominance of *Alphaproteobacteria* methanotrophs in low-N ecosystems could perhaps be linked to their general lack of *haoAB* genes and their higher expression of nitrogen fixation genes relative to the *Gammaproteobacteria* (Aronson et al. 2013; Bodelier and Steenbergh 2014). However, this linkage of community structure to specific gene content, or expression of genes, has not yet been validated.

Robust correlations between HAO expression in methanotrophs and relative inhibition by ammonium have yet to be fully investigated; however, it is clear that ammonium is only sometimes inhibitory and is often a stimulant for methane consumption in culture and in the environment (Bodelier et al. 2000; Bodelier and Laanbroek 2004; Nyerges and Stein 2009; Bodelier and Steenbergh 2014). It should be noted, though, that the complex interplay between plants, microbes, and nutrient availability is more critical in determining the relative strength of biologically driven methane sinks than N-input alone.

4.4 Nitrogen Oxides in Dissimilatory Single-Carbon Metabolism

The discovery of nitrite-dependent anaerobic methane oxidation (n-DAMO) by bacteria in the NC10 phylum was paradigm-shifting as this was the first report of a free-living bacterium capable of generating intracellular O_2, from dismutation of NO, to oxidize methane (Ettwig et al. 2010). This metabolism is garnering great interest in wastewater treatment as a solution to removing methane and nitrite to N_2

while bypassing the production of N_2O (Bhattacharjee et al. 2016; Ding et al. 2017). Thus far, NC10 bacteria have been detected using molecular approaches in marine oxygen minimum zones (Padilla et al. 2016), freshwater sediments (Long et al. 2017), soils (Shen et al. 2016; Vaksmaa et al. 2016), and wastewater treatment plants (Zhu et al. 2017), among others. The hypothesis is that NC10 bacteria should be abundant in any anoxic ecosystem where nitrite and methane are generated and both substrates are equally accessible. There are studies emerging that show strong associations of NC10 bacteria with other microbes; for instance, a nitrate-reducing ANME archaeon was co-cultivated in the original NC10 enrichment as the provider of nitrite (Welte et al. 2016).

Although intriguing, the presence and activity of gammaproteobacterial methanotrophs often eclipse that of NC10 bacteria in suitable anoxic ecosystems (Mackelprang et al. 2011; Tavormina et al. 2013; Skennerton et al. 2015; Oswald et al. 2016). Genome analysis of gammaproteobacterial methanotrophs revealed a high occurrence of genes encoding nitrite- and nitric oxide reductases, whereas alphaproteobacterial methanotrophs are largely devoid of these genes (Stein and Klotz 2011). Physiological studies of several gammaproteobacterial methanotroph species revealed that these reductases are induced under low oxygen to reduce nitrite to NO to the final product, N_2O (Hoefman et al. 2014; Kits et al. 2015b). When an electrogenic nitrate reductase (NarG) is expressed along with nitrite- and nitric oxide reductase, as in *Methylomonas denitrificans* FGJ1, methanotrophs can utilize nitrate as an alternative electron acceptor to oxygen, also producing N_2O as the final product (Kits et al. 2015a). Methylotrophic bacteria, such as *Methylotenera mobilis*, encode a similar denitrification pathway to *M. denitrificans* FGJ1 and produce N_2O by coupling the oxidation of methanol to the reduction of nitrate, although in this case, oxygen depletion does not appear to be a strong regulator of this activity unlike in *M. denitrificans* FJG1 (Mustakhimov et al. 2013). Thus, the niche preference for denitrifying methanotrophs and methylotrophs could potentially be dictated by oxygen level.

The main difference between gammaproteobacterial and NC10 methanotrophs is that the former still requires externally supplied O_2 to activate methane oxidation; however, they appear to be superb O_2 scavengers. It is hypothesized that a variant of pMMO expressed by the *pxmABC* operon has a high affinity for O_2 that enables methane activation at exceedingly low O_2 concentrations (Tavormina et al. 2011; Kits et al. 2015a, 2015b). Under these conditions, the bacteria respire nitrate instead of O_2 and/or ferment (Kalyuzhnaya et al. 2013) to support hypoxic methane oxidation. It remains to be demonstrated whether there is a strong correlation between the presence and expression of *pxmABC* with methanotroph-encoded denitrification genes in hypoxic to anoxic ecosystems, although supportive studies are emerging (Tavormina et al. 2013).

4.5 Methanotrophs and Methylotrophs as Versatile Players in a Complex N Cycle

As described above, nitrogen is a key regulator of methanotrophy and methylotrophy in both assimilatory and dissimilatory metabolism. There is a substantial body of literature attempting to untangle the various roles that nitrogen plays in regulating methanotrophy and methylotrophy in many ecosystems, including terrestrial, aquatic, sedimentary, agricultural, marine, and engineered ecosystems (Bodelier and Laanbroek 2004; Bodelier and Steenbergh 2014; Chistoserdova 2015). The ability of methanotrophs to oxidize ammonia to nitrite links their activity to nitrification, although the general consensus is that methanotrophs are not competitive with ammonia-oxidizing microorganisms as they lose energy from co-metabolism and cannot grow on ammonia as a sole energy source. The conflicting data discussed above, that ammonium is sometimes inhibitory and sometimes stimulatory to the methane sink, is highly dependent on the environment, community composition, and experimental conditions of the particular study. Furthermore, the confounding variables of plant–microbe associations and complex interplay between organisms and nutrient acquisition require a systems-level view of how methanotrophs interact within a community (Bodelier and Steenbergh 2014; Ho et al. 2016).

It is not yet clear how ubiquitous denitrifying proteobacterial methanotrophs and methylotrophs are in hypoxic or anoxic ecosystems, but ongoing studies are building the case for niche preference by particular bacterial groups and with particular gene content (Beck et al. 2014; Chistoserdova 2014; Skennerton et al. 2015; Oswald et al. 2016). It is anticipated that more linkages will be made between denitrifying proteobacterial methanotrophs and methylotrophs in meta-omic datasets now that the physiology has been described and validated for several genes and functional modules.

4.6 Nitrogen as a Regulator of Single-Carbon Bioconversions

Industrialization of methanotrophs and methylotrophs requires an understanding of how nitrogen regulates cellular growth and directionality of metabolites into commercially useful products. One of the primary determinants for successful scale-up of any microbial culture is a suitable nitrogen source that is both inexpensive and supportive of growth, metabolism, and product accumulation. Since methanotrophs and methylotrophs are most commonly grown with ammonium or nitrate, these are the most common N-sources for industrial production and scale-up of biomass. However, as discussed above, the effect of ammonium and nitrate is variable from strain to strain in terms of inhibition and toxicity; hence, the N-source must be determined empirically for each strain and each commercial product.

The production of polyhydroxybutyrate (PHB) by methanotrophs and methylotrophs is the best-studied bioindustrial target in terms of N-source effects on production. It is well known that induction of PHB biosynthesis by microbes requires a starvation signal, usually accomplished by limiting a nutrient, often

nitrogen, while providing carbon in excess (Lee 1996). Comparison of PHB induction has been empirically investigated in a number of *Alphaproteobacteria* methanotroph strains by limiting ammonium or nitrate or by growing bacteria diazotrophically (Pieja et al. 2011a; Zhang et al. 2017). Although there is considerable strain-to-strain variation, it is clear that only *Alphaproteobacteria* methanotrophs and methylotrophs that express the serine cycle for formaldehyde assimilation are capable of synthesizing PHB, whereas the *Gammaproteobacteria* methanotrophs that utilize the RuMP pathway lack this capacity (Pieja et al. 2011b). Few studies have compared the production of PHB as a direct function of growth, and eventual starvation, with a specific N-source (Rostkowski et al. 2013; Zhang et al. 2017). However, even these few studies show distinctive strain-based preferences for N-source in production of PHB. These studies strongly indicate that industrialization of methanotrophs and methylotrophs will require extensive strain-by-strain characterization to determine the optimal N-source and molecular mechanisms underlying metabolic regulation by N-source.

4.7 Perspectives and Future Outlook

The long-held view of proteobacterial methanotrophs as obligate, aerobic, single-carbon utilizers has been debunked with the discoveries of fermentative pathways (Kalyuzhnaya et al. 2013), multicarbon substrate utilization (Dedysh et al. 2005), and more recently, nitrate respiration (Kits et al. 2015a). Both methanotrophs and methylotrophs assimilate a diversity of N-sources; however, there remain strong strain-to-strain variations in N-utilization that are not often linked to specific phylogenetic lineages. That said, genome sequencing has revealed that N-fixation genes and modules are more highly represented in *Alphaproteobacteria* than in *Gammaproteobacteria*, perhaps explaining the greater dominance of *Alphaproteobacteria* methanotrophs and methylotrophs in low-N ecosystems and in association with plants. Conversely, denitrification genes appear more commonly in *Gammaproteobacteria* methanotrophs, which could explain their presence, and relatively high abundance, in hypoxic to anoxic ecosystems where methane, nitrate/nitrite, and nitrous oxide co-occur (Mackelprang et al. 2011; Tavormina et al. 2013; Oswald et al. 2016).

Future applications of methanotrophs and methylotrophs to bioindustry will require a greater understanding of how nitrogen regulates metabolic processes, perhaps through a combination of multi-omics technologies. Aside from characterizing and optimizing nitrogen usage and efficiency of individual bacterial strains, a more comprehensive goal is to understand how nitrogen affects interactions of methanotrophs and methylotrophs in complex communities, including those with plants and animals. We are beginning to see more robust linkages between methanotrophs and methylotrophs with N-cycle processes, including co-occurrence and expression of gene sets and modules (e.g., *pxmABC* with denitrification genes). Future research must validate and expand the connections between single-carbon and nitrogen metabolism, as this interplay governs a large segment of microbial physiology, microbial ecology, and ecosystem function.

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Metals in Methanotrophy

5

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5.1 Copper (Cu)

Cu is one of the most studied and most important micronutrients known to control methanotrophic growth (Glass and Orphan 2012). The first step during methanotrophy requires the oxidation of methane to methanol, a step that requires the activation of the very strong C–H bond of methane (105 kcal/mol) (Blanksby and Ellison 2003) and is catalyzed by methane monooxygenases (MMOs). Two different forms of MMOs can catalyze this oxidation step: the membrane-associated or particulate methane monooxygenase (pMMO) and the soluble and cytoplasmic MMO (sMMO).

5.1.1 pMMO

pMMO is found in most known aerobic methanotrophs except for some members of the genus *Methylocella* (Hanson and Hanson 1996). Molecular details of the active center remain unclear, including metal composition, as the recovery of the active pure form has been inefficient (Semrau et al. 2010). pMMO is a heterotrimer, where each subunit is composed of three peptides with molecular masses of 45 KDa (α-PmoB), 26 KDa (β-PmoA), and 23 KDa (γ-PmoC) (Zahn and DiSpirito 1996; Basu et al. 2003; Choi et al. 2003; Lieberman and Rosenzweig 2005). pMMO is found in intracytoplasmic membranes which serve to increase the surface area for pMMO. The N-terminus of PmoB is localized to the periplasm, whereas the N-termini of PmoA and PmoC are cytoplasmic. A truncation containing two soluble

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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_5

PmoB subdomains joined by a flexible linker region was shown to exhibit methane oxidation activity (Balasubramanian et al. 2010) suggesting the di-Cu center coordinated by PmoB constitutes the active site of pMMO (Lieberman and Rosenzweig 2005; Balasubramanian and Rosenzweig 2007; Hakemian et al. 2008; Balasubramanian et al. 2010). Electron paramagnetic resonance (EPR) analysis of pMMO from *Methylococcus capsulatus* Bath showed a di-Cu (I–II) resting state in the dinuclear active site of PmoB. Additionally, the PmoC subunit coordinates a mono-Cu site and can coordinate zinc, but no evidence of a role for PmoC in catalysis has been shown (Culpepper et al. 2014). Further, the presence of an Fe center in pMMO (coordinated by both PmoA and PmoC and resembling the Fe center of sMMO) has also been shown after purification attempts and UV-visible spectrophotometric analysis (Zhan and DiSpirito 1996). Zhan et al. has also proposed a cytochrome bc_1 complex containing three heme groups and one [2Fe-2S] cluster as the electron donor to pMMO (Zhan and DiSpirito 1996). Recent Mössbauer studies are consistent with these observations as pMMO activity can been correlated with Fe concentration (Martinho et al. 2007; Semrau et al. 2010). Further optimization of the purification of active pMMO would allow for detailed structural, molecular, and kinetic analyses. A proposed mechanism favoring a di-Cu active site considers that a di-Cu (I–II) center is reduced to the di-Cu (I–I) state. Oxygen reacts with the reduced center to either form a reactive bis (μ-oxo) di-Cu (II–III) or (μ-oxo) (μ-hydroxo) di-Cu (II–III) species that is capable of methane oxidation as shown in Fig. 5.1 (Shiota et al. 2013; Shiota and Yoshizawa 2009; Yoshizawa and Shiota 2006). However, Cu (III) has not been observed in biological systems, so further experimentation is necessary to corroborate this proposed mechanism. In addition to the resting state, a mechanism in which a tyrosine residue donates an H atom to the μ-η^2:η^2-peroxoCu (II–II) structure leading to the formation of the (μ-oxo) (μ-hydroxo) di-Cu (II–III) has been calculated using time-dependent density functional theory (TD-DFT) studies as shown in Fig. 5.1b (Itoyama et al. 2016). The physiological reducing agent for pMMO has not definitively been identified. There is

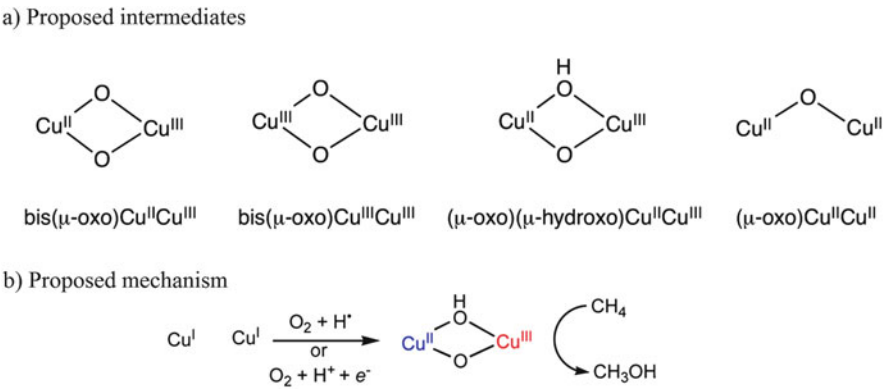

Fig. 5.1 Schematic representation of the catalytic mechanism of pMMO. (**a**) Intermediates and (**b**) reaction (modified from Shiota et al. 2013)

precedent for electron passage from cytochromes (cytochrome *c*) to an oxygen-binding site mediated by a functional protein subunit containing a dinuclear Cu center, as is the case for the subunit II, Cu_A of the aa_3-type cytochrome *c* oxidase from *Thiobacillus versutus* raising the possibility of a similar mechanism for pMMO (Dennison et al. 1995). Alternatively, electrons from methanol oxidation, catalyzed by alcohol dehydrogenases such as MxaFI, XoxF, or ExaF, can be transferred to reduce a cytochrome *c* which can then reduce the pMMO active site (Leak and Dalton 1986; Tonge et al. 1975). Protein-protein interactions between pMMO, specifically PmoB, and methanol dehydrogenase have been described (Culpepper and Rosenzweig 2014; Myronova et al. 2006). Ubiquinol has also been suggested as a potential reducing agent generated by a type 2 NADH:quinone oxidoreductase (NDH-2) (Shiemke et al. 2004; Choi et al. 2003; Cook and Shiemke 2002). NADH is unable to reduce purified pMMO, yet when added in cell free extracts, reduction of pMMO occurs. This result suggests that NADH can induce activity of the NDH-2 complex and/or the Na^+-translocating NADH-quinone reductase (NQR) and/or the formaldehyde-quinone reductase (FQR) to produce the quinone pool necessary for pMMO activation. In fact, it has been shown that adding NDH-2 in vitro increases pMMO activity upon addition of NADH (Choi et al. 2003). However, there is no evidence that pMMO and NDH-2 form a stable complex nor has a quinol binding site been identified in pMMO.

5.1.2 Copper Uptake

To obtain Cu from the environment for pMMO activity, many characterized methanotrophs excrete methanobactins (Mbns), modified polypeptide chalkophores with high affinity for Cu (DiSpirito et al. 2016; Behling et al. 2008; Kim et al. 2005, 2004). Production of these Mbns and their transport systems is controlled by Cu at the level of gene expression and includes MbnA (Kenney et al. 2016); the Mbn precursor (Semrau et al. 2013); MbnM, a multidrug exporter family member proposed to export Mbn (Kenney and Rosenzweig 2013); MbnT (Gu et al. 2016a, b; Dassama et al. 2016; Kenney and Rosenzweig 2013), a TonB-dependent receptor; MbnE (Dassama et al. 2016), a periplasmic Mbn binding protein; and additional less characterized genes required for Mbn synthesis (Dassama et al. 2016). In the environment, it is likely that methanotrophs pirate Mbns from other neighboring methanotrophs, and it has been proposed that these Mbns act as signaling molecules to facilitate community interactions (Farhan Ul Haque et al. 2015).

5.1.3 The Cu Switch

Methanotrophs that contain both MMO forms are susceptible to the "Cu switch": changes in relative expression of sMMO based on Cu/biomass ratios (Nielsen et al. 1997; Murrell et al. 2000). Ratios of 5.64 μmol Cu g^{-1} protein favor enrichment of pMMO, and sMMO activity is no longer detected (Morton et al. 2000). In

$$\Delta = \frac{\nu_G - \sum_{i=1}^{n} \nu_{P_i}}{\nu_G} = \frac{\frac{V_{\max}^{G} * S^{G}}{K_S^{G} + S^{G}} - \sum_{i=1}^{n} \frac{V_{\max}^{P_i} * P_i}{K_s^{P_i} + P_i}}{\frac{V_{\max}^{G} * S^{G}}{K_S^{G} + S^{G}}}$$

Fig. 5.2 Mathematical equation to determine ratios of sMMO and pMMO (Semrau 2011)

Methylosinus trichosporium OB3b and *M. capsulatus* Bath, transcription of the first gene in the *smmo* gene cluster, *mmoX*, occurs from a σ^N-dependent promoter suggesting that nitrogen availability may play a key role in the ability of cells to oxidize methane in the absence of copper (Stafford et al. 2003; Csaki et al. 2003). In these same organisms, two genes adjacent to the *smmo* operon, *mmoR* (σ^N-dependent transcriptional regulator) and *mmoG* (a GroEL homolog hypothesized to facilitate proper folding of MmoR), are required for expression of the *smmo* genes (Stafford et al. 2003; Csaki et al. 2003). Very little is known about the mechanism controlling the Cu switch or pMMO expression. However, in *M. trichosporium* OB3b, MmoD, a protein of unknown function encoded in the *smmo* operon, was shown to be required for the switch to occur (Semrau et al. 2013). Further, no *smmo* transcription or sMMO activity was observed from cultures of a strain lacking *mmoD*. Additionally, in *M. capsulatus* Bath, Cu can also be obtained via MopE, a membrane-bound Cu-binding protein or via a truncated form of MopE which is secreted from the cell to bind oxidized and reduced Cu (Helland et al. 2008; Karlsen et al. 2003).

While pMMO has a higher affinity for methane than sMMO, it has a significantly slower methane oxidation rate. As copper levels regulate production of both enzymes, it is feasible to suggest that pMMO and sMMO ratios can be tunable and that copper can enhance either sensitivity or productivity (via increased rate) of methane oxidation. A mathematical model (Δ model) has been used to predict this fine tuning (Fig. 5.2). This ability to tune the Cu switch has important implications for the effectiveness of using methanotrophs to degrade pollutants in the bioremediation industry and to produce value-added chemicals from methane (Semrau 2011; Lee et al. 2006).

5.1.4 Cytochromes

These Fe-containing enzymes can be found as monomeric proteins or as subunits of large protein complexes. In methanotrophy, cytochromes are essential components that recycle electrons to catalyze substrate oxidations to be used for energy production. Electron transfer between pMMO and a cytochrome *bc1* complex containing three heme groups and one [2Fe-2S] cluster has been demonstrated in vitro (Zhan and DiSpirito 1996). Further, methanol dehydrogenases (MDHs), such as MxaFI and XoxF, oxidize methanol to formaldehyde using two c-type cytochromes (*c*L and *c*H) as electron acceptors (Hanson and Hanson 1996). These cytochromes are then

oxidized by the cytochrome *aa3* complex, which contains two hemes and two Cu atoms (Hanson and Hanson 1996), and has been proposed to be one of the four different terminal oxidase systems present in methanotrophs (Semrau et al. 2010).

Intriguingly, in the methanotroph *M. capsulatus* Bath, transcriptomic profiling showed that in addition to the genes encoding pMMO and sMMO, 21 *c*-type cytochromes along with multiple ion transport genes were differentially expressed by Cu suggesting Cu plays an integral role in controlling energy metabolism (Larsen and Karlsen 2016). Examples of the Cu-induced cytochromes include the *c*553o family of multiheme *c*-type cytochromes; the di-hemes SACCP, MCA0424, and MCA0426; and cytochromes MCA1185, MCA1186, and MCA1187. Sequence and structural analysis of MCA1187 suggest that this protein may be a member of a novel family of cytochromes (characterized as a cytochrome cbd) and is the terminal cytochrome c oxidase. Further, the majority of these overexpressed *c*-type cytochromes in sMMO-producing cells were located at the cell surface according to LTQ-ORBITRAP MS studies suggesting that *M. capsulatus* Bath has the ability to utilize extracellular electron acceptors (Larsen and Karlsen 2016) and opens an emerging field for biotechnological applications using electrode-bacteria interactions (Richardson et al. 2012).

5.2 Iron (Fe)

5.2.1 sMMO

Unlike pMMO, sMMO has been extensively biochemically characterized. sMMO is a cytoplasmic and soluble MMO that is comprised of three units: a regulatory protein (MMOB), a dimeric hydroxylase (MMOH) with a di-Fe center (251 kDa $\alpha_2\beta_2\gamma_2$), and a reductase subunit (MMOR) with a [2Fe-2S] cluster ferredoxin domain, a NADH binding site, and a FAD-binding domain (Chatwood et al. 2004; Müller et al. 2002; Lipscomb 1994; Fox et al. 1989; Colby and Dalton 1979). sMMO belongs to the bacterial multicomponent monooxygenase (BMM) superfamily of proteins. Methane oxidation using sMMO is NADH dependent, and the mechanism and intermediates formed during the catalytic cycle have been described (Sirajuddin and Rosenzweig 2015; Sazinsky and Lippard 2015). Briefly, the di-Fe (III–III) center of MMOH (resting state) is reduced by MMOR in two sequential electron transfers to the di-Fe (II–II) state (Wang et al. 2015). Oxygen reacts with reduced MMOH to form an intermediate O, followed by the peroxo intermediates P*, a di-Fe (II–II) species, and P, a peroxo-bridged di-Fe (III–III) species that is converted to the di-Fe (IV) intermediate Q (Fig. 5.3). Intermediate Q complexes with methane resulting in the formation of the product complex, T (Sazinsky and Lippard 2015). Interactions between the ferredoxin (MMOR) and MMOH components have been observed crystallographically for other members of the BMM superfamily such as the toluene 4-monooxygenase, but intriguingly toluene monooxygenases are not able to hydroxylate methane potentially due to structural differences (Acheson et al. 2014). MMOB is known to complex with MMOH leading to a reaction rate increase

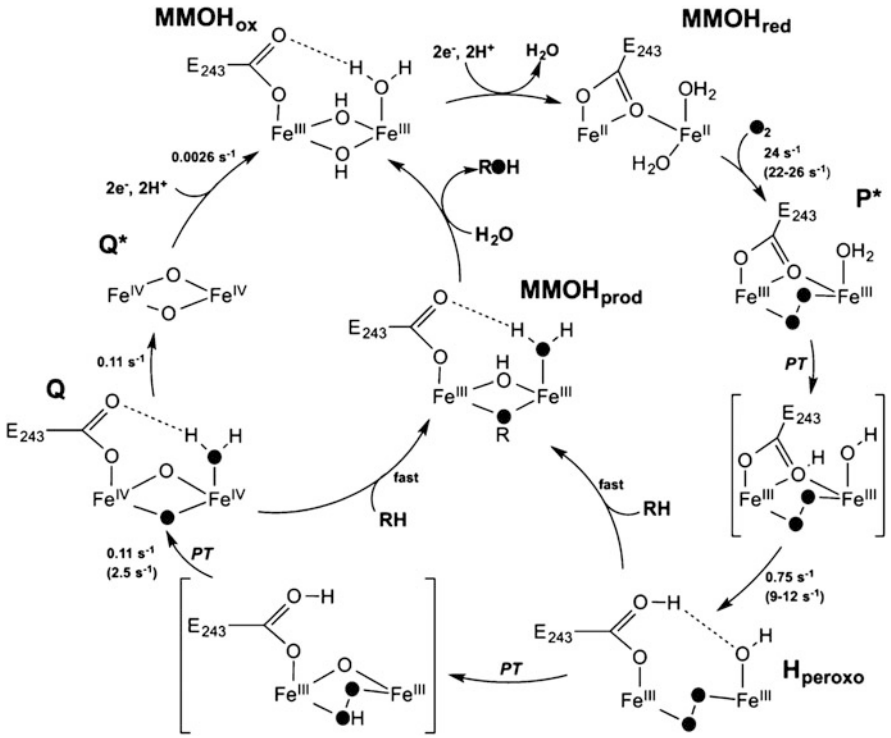

Fig. 5.3 Schematic representation of the catalytic mechanism of sMMO (Sazinsky and Lippard 2015)

of two- to threefold by accelerating reaction intermediate formation as well as O_2 and proton delivery to the active site (Liu et al. 1995; Fox et al. 1989, 1991). A reduction of the potential of the di-Fe center of MMOH by the complex formation MMOB and MMOH was previously suggested, but fluorescence anisotropy and double electron-electron resonance (DEER) studies have shown that MMOB binds predominantly $MMOH_{red}$ (Wang and Lippard 2014; Lee et al. 2013). Further, crystal structure analysis of *M. capsulatus* (Bath) has shown that the MMOH-MMOB interaction opens a substrate channel to the Fe center by repositioning MMOH residue Phe188, which gates the hydrophobic cavities and solves the important challenge of substrate to the active site (Lee et al. 2013; Rosenzweig et al. 1997). Together, sMMO activity relies on the formation of the three-piece complex (MMOH, MOOB, MOOR), and biotechnological efforts to enhance methane oxidation have centered on changes in expression levels of each component of the complex (Lawton and Rosenzweig 2016).

Little is known about the different iron uptake systems in methanotrophic bacteria; however, it has been shown that *M. trichosporium* OB3b produces siderophores as the production of an Fe-chelating, fluorescent compound was observed in solid medium (Yoon et al. 2010). However, the siderophore structure and pathway for its

biosynthesis are not known. In Gram-negative bacteria, the siderophore-Fe (III) complex is transported into the cell through a TonB-dependent receptor located on the cell surface, complexed with a periplasmic binding protein, and, using the TonB-ExbBD energy transduction system, is translocated through the periplasmic space to an ABC transporter. Fe (III) is then transported into the cytoplasm either by itself or bound to the siderophore where it is then released and available for use (Miethke and Marahiel 2007; Lankford and Byers 1973). Transcriptomic studies from *M. trichosporium* OB3b grown on methane are consistent with this observation, showing upregulation of genes homologous to those encoding the biosynthetic pathway, excretion, uptake, and regulatory systems for pyoverdine. (Matsen et al. 2013).

5.3 Lanthanides (Ln)

Lanthanides (Ln), also known as 4f elements, consist of 15 elements from lanthanum (Z=57) to lutetium (Z=71). They are relatively abundant in the Earth's crust, but they exhibit low aqueous solubility such that Ln are also known as "rare earth elements" due to their limited biological accessibility. Their electrons have a partial occupation of the 4f shell, and all have high coordination numbers and redox chemistry. Ln are strong Lewis acids that coordinate with hard bases (carboxylates) and highly electronegative donors such as nitrogen or oxygen (Lim et al. 2009). In methanotrophy, Ln are known to complex with the coenzyme pyrroloquinoline quinone (PQQ) and are thought to enhance catalysis of diverse alcohol dehydrogenases as they are potent Lewis acids (Keltjens et al. 2014).

5.3.1 XoxF

xoxF-like genes are found in many methylotrophic genomes but also in genomes from microorganisms that are not known to be able to oxidize methanol (Vorobev et al. 2013). The second step in methanotrophy, the oxidation of methanol to formaldehyde, was thought to be solely catalyzed by the well-characterized calcium (Ca)-dependent MxaFI-MDH. The role of Ca in the MxaFI-MDH is proposed to function as a Lewis acid to polarize the C_5–O_5 bond in PQQ facilitating the oxidation of methanol. The most accepted mechanism proposes a base-catalyzed nucleophilic addition of methanol to the C_5 carbonyl group of PQQ facilitated by an aspartate and leading to production of formaldehyde, followed by the deprotonation of the catalytic aspartate residue as shown in Fig. 5.4 (Prejanò et al. 2017; Leopoldini et al. 2007). $PQQH_2$ is formed after tautomerization and enables a two-step transfer of two electrons to the cytochrome c_L (Davidson 2004). The role of XoxF in methylotrophy was puzzling for some time, as XoxF shares more than 40% identity with the large subunit of the Ca-MDH, MxaFI, yet when first purified from *M. extorquens* AM1, XoxF had an activity too low to support methanol growth (Schmidt et al. 2010). However, when Ln were added to the growth medium, it was shown that XoxF and not MxaFI catalyzed the oxidation of methanol (Hibi et al. 2011). XoxF purified

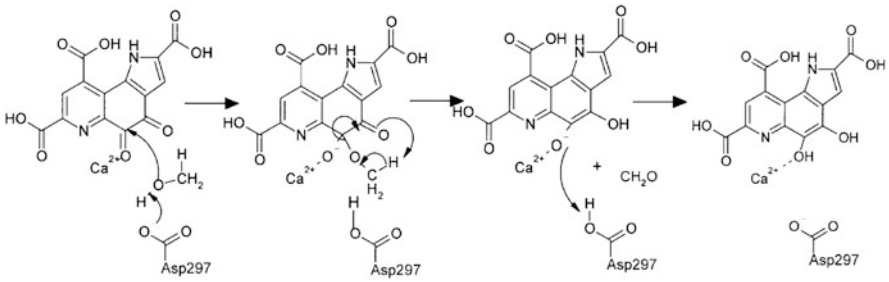

Fig. 5.4 Schematic representation of the catalytic mechanism of Ca- or Ln-PQQ-containing methanol dehydrogenases (taken from Leopoldini et al. 2007)

from *M. extorquens* AM1 (Nakagawa et al. 2012) and *Methylacidiphilum fumariolicum* SolV has been shown to contain Ln such as lanthanum (La (III)) and cerium (Ce (III)) which are tightly bound in the active site (Pol et al. 2014). Diverse Ln (from La to samarium (Sm)) can support growth with methanol suggesting that Ln binding to XoxF is somewhat promiscuous (Vu et al. 2016; Chu and Lidstrom 2016). XoxF homologs are very divergent, but they all share with MxaF, the amino acids known to bind methanol, PQQ, and calcium in the active site. However, an aspartate (D301 numbering according to *M. fumariolicum* XoxF structure) replacing an alanine is conserved in all XoxF homologs (Keltjens et al. 2014). Interestingly, the same aspartate is present in the recently characterized Ln-dependent ethanol dehydrogenase ExaF, further suggesting that this aspartate is necessary for coordination of Ln (Good et al. 2016). Detailed studies to define the role of Ln in catalysis is undergoing, but evidence that Ln act as potent Lewis acids and "activate" PQQ similar to the role of calcium in MxaFI has been reported (Fig. 5.4). Density functional theory (DFT) analysis from *M. fumariolicum* XoxF determined that the most stable redox form for the coordination between Ce and PQQ was the Ce (III)-PQQ (0) state and estimated that the Ce (III) oxidation potential had a value of +1.35 V versus saturated calomel electrode (SCE) (Bogart et al. 2015). It has been suggested that Ln-containing alcohol dehydrogenases may have higher oxidation capacities as they are more potent Lewis acids than their Ca counterparts. DFT studies comparing Ca- versus Ce-containing XoxF from *Methylophilus methylotrophus* and *M. fumariolicum* respectively not only corroborated an addition-elimination, protonation mechanism to oxidize methanol but also shown that Ce (III) thermodynamically stabilizes the nucleophilic agent. As a result, the potential energy surface obtained from Ce-containing XoxF is different from the Ca-containing one (Prejanò et al. 2017). In vitro studies suggest the oxidation to the corresponding aldehyde is not the final product and, instead, the aldehyde can be further oxidized to the corresponding acid. This effect has been shown with ExaF from *M. extorquens* AM1 for ethanol (Good et al. 2016) and with XoxF purified from *M. fumariolicum* SolV for methanol (Pol et al. 2014), each producing acetate and formate, respectively. Yet, formaldehyde is a key intermediate for reducing power production during methanotrophic and methylotrophic growth. Oxidation of

formaldehyde via the GSH-dependent pathway or the H_4MPT-dependent pathway leads to the production of NAD(P)H. Direct oxidation to formate by XoxF may have implications in terms of anabolism/catabolism and energy/reducing power production that might need to be balanced by yet unknown mechanisms. It is also possible that in vivo XoxF produces formaldehyde exclusively or can produce both formaldehyde and formate based on cellular needs. Detailed genetic and biochemical studies are needed to clarify this issue. If formate is directly produced from methanol in vivo, then metabolomics along with carbon flux analysis may shed light as to how the cell maintains its reducing power pools.

Due to the insolubility of Ln, how methanotrophs and methylotrophs efficiently sense, solubilize, and transport these metals is an active area of interest that is yet largely unexplored. While it is unknown how Ln are acquired from the environment, the acquisition systems are likely sensitive and efficient (Vu et al. 2016). Work in *M. extorquens* AM1 using a strain that lacks the Ca-dependent MDH has even been shown to obtain Lns from computer hard drive magnets and use them for growth. The hard drive magnet's metal composition included on average (% total weight) 63% Fe, 3% Co, 1% B, 0.3% Al, and 0.1% Cu and the Ln composition of 24% neodymium (Nd) and 2% praseodymium (Pr). None of the additional transition metals tested allowed growth of the mutant, demonstrating that growth was Ln-dependent (Martinez-Gomez et al. 2016). The ability of methanotrophs and methylotrophs to solubilize Ln opens avenues to engineer these microorganisms as platforms for biotechnology, considering that Ln are critical metals for the vast majority of the technological devices in our everyday life.

5.3.2 The Ln Switch

Analogous to the Cu switch, differential expression of the Mxa and XoxF MDHs is controlled by the presence of specific Ln. In organisms studied thus far, exogenous concentrations of <1 μM La, Ce, Pr, or Nd are sufficient to repress *mxa* expression and induce *xoxF* expression suggesting that in the environment, the Ln-dependent MDH is preferred over the Ca-dependent MDH (Vu et al. 2016; Chu and Lidstrom 2016; Gu et al. 2016a; Farhan UI Haque et al. 2015). To date, Ln-dependent growth and expression appears limited to these four Ln with severely limited growth and mediation of the Ln switch also facilitated by samarium (Sm) in a few organisms (Vu et al. 2016; Chu and Lidstrom 2016; Gu et al. 2016a; Farhan UI Haque et al. 2015). Very little is known about the mechanisms controlling the Ln switch. In the methylotroph, *M. extorquens* AM1, the MxbDM two-component system is required for the switch along with the *xoxF* gene itself (Vu et al. 2016). However, in *Methylomicrobium buryatense* which lacks MxbDM, the Ln switch requires the histidine kinase MxaY and the orphaned response regulator MxaB and does not require the presence of the *xoxF* gene (Chu et al. 2016; Chu and Lidstrom 2016). The effect of Cu on the Ln switch has been studied in type I and type II methanotrophs. Intriguingly, the effect of Cu on the Ln switch differs depending on the methanotroph studied. In the type I methanotroph, *M. buryatense*, Cu presence has very little effect

on expression of the *mxa* and *xoxF* genes (Chu and Lidstrom 2016). However, in the type II methanotroph, *M. trichosporium* OB3b, exogenous Cu dampens the effect of Ln on the switch allowing expression of the Mxa-MDH even when Ln are present (Gu et al. 2016a, b; Farhan UI Haque et al. 2015). It has been proposed that the various mechanisms that contribute to expression of the *mxa* and *xoxF* genes are numerous and complex and likely involve regulatory cascades (Vu et al. 2016; Chu et al. 2016; Chu and Lidstrom 2016). There is much left to learn regarding the regulation of the *mxa* and *xox* genes, and the mechanism of Ln acquisition and transport has yet to be explored. A deeper understanding of these processes may facilitate the engineering of methanotrophs and methylotrophs to better function as environmental platforms for the recovery of rare earth elements from electronics waste.

5.4 Cobalt (Co)

Methylotrophs using the serine cycle for carbon assimilation depend on glyoxylate regeneration. The ethylmalonyl-CoA (EMC) pathway regenerates glyoxylate by catalyzing two carboxylation steps. Two enzymes that are part of this cycle, methylmalonyl-CoA mutase (Mcm) and ethylmalonyl-CoA mutase (Ecm), require cobalamin (Vitamin B_{12}) as a coenzyme (Erb et al. 2007). Cobalamin is Co-dependent and therefore it has been suggested that availability of Co could affect flux through the EMC pathway. Under growth conditions where Co is limiting, the intermediates ethylmalonyl-CoA and methylmalonyl-CoA accumulate between 6- and 35-fold when compared to non-limiting conditions (Kiefer et al. 2009). Interestingly, long-term evolution studies of *M. extorquens* AM1 populations grown in methanol medium evolved an increase in Co uptake by upregulating two genes: *icuA*, which has similarity to TonB-dependent receptors, and *icuB*, a gene of unknown function (Chou et al. 2009). This finding prompted the development of a new growth medium for culturing *M. extorquens* (Delaney et al. 2013).

Different concentrations of Co in the growth medium appear to affect carbon distribution through different carbon assimilation pathways. One study showed that when using two different Co concentrations (low, 1.35 μM, and high, 6.31 μM, respectively), flux distribution through the EMC was the same. Instead, changes in flux distribution were apparent between the serine cycle, the partial TCA cycle, and the pentose phosphate pathway, particularly in redundant steps for C3-C4 interconversions (Fu et al. 2016). The mechanism(s) for these changes are not known. Biotechnologically, the understanding of the Co effect on carbon flux through the EMC pathway is of great importance as several intermediates of the EMC pathway are precursors of value-added products (Hu and Lidstrom 2014). An emerging field includes the production of the EMC-derived dicarboxylic acids, specifically mesaconic acid and (2S)-methylsuccinic acid for production of bioplastics and food additives (Lee et al. 2002; Werpy and Petersen 2004). It has been shown that a balance between accumulation of the CoA ester intermediates and growth can be achieved by overproducing the thioesterase, YciA. When Co concentrations were reduced to 0.2 μM in the medium (12-fold decrease compared

to non-limiting conditions), a sixfold increase in accumulation of the desired precursors was observed (Sonntag et al. 2014).

5.5 Molybenum (Mo) and Tungsten (W)

5.5.1 FDHs

The oxidation of formate to CO_2 is central to methanotrophic organisms that use the serine cycle for assimilation as this oxidation step is usually linked to NADH production providing reducing equivalents for the cell and the electron transport chain for ATP synthesis. In organisms oxidizing formaldehyde via the RuMP cycle, the formate oxidation step is less critical and formate dehydrogenase (FDH) activity is typically not high (Chistoserdova 2011; Anthony 1986). FDH occurrence in methanotrophs and methylotrophs is not uniform, but the majority of prokaryotic FDHs coordinate Mo in their active site. Some methanotrophs also have W-containing FDHs that are mainly found in organisms that live under extreme conditions. Mo- and W-containing FDH homologs are very divergent and are usually a cytoplasmic heteromultimeric complex of proteins. However, the active site of FDH is conserved and includes a His residue, a SeCys residue, the metal cofactor (Mo or W), and an [Fe-S] cluster. Several mechanisms for catalysis have been proposed, but one that is consistent with kinetic and DFT analysis suggests that the Mo (IV) or W (IV) resting state is coordinated via sulfur ligands by a Cys and a SeCys residue. Upon binding of formate, the SeCys bond is displaced enabling the abstraction of the proton from formate. Two electrons are transferred to Mo/W and CO_2 is released. The proton is then transferred to a His residue, and reoxidation of Mo/W occurs via the [Fe-S] present in the active site as shown in Fig. 5.5 (Maia et al. 2015).

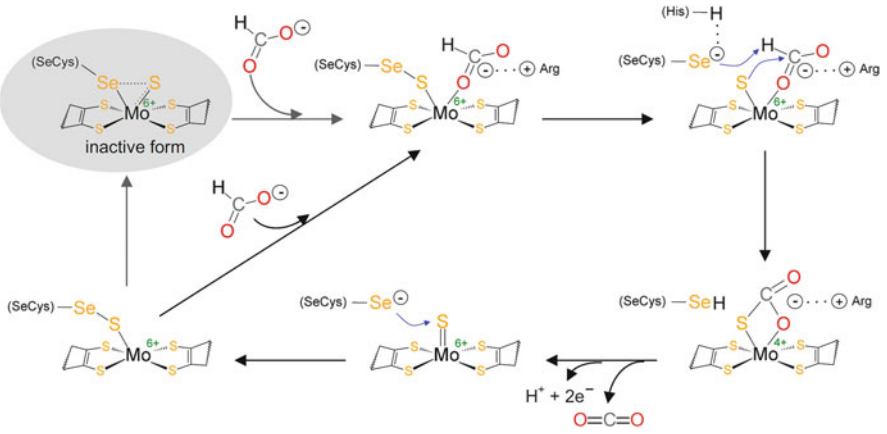

Fig. 5.5 Schematic representation of the catalytic mechanism of formate dehydrogenase (taken from Maia et al. 2015)

There is a growing interest in using formate as a liquid transportable intermediate for the storage of H_2 or CO_2 (Watkins and Bocarsly 2014). Although the reactions described in this section describe the oxidation of formate to CO_2, understanding the catalytic mechanism and the role of the cofactors can help design protein engineering strategies for FDHs that can catalyze the reverse reaction.

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Pyrophosphate-Dependent Enzymes in Methanotrophs: New Findings and Views

6

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Abbreviations

F6P	fructose-6-phosphate
FBP	fructose-1,6-bisphosphate
PFK	phosphofructokinase
PEP	phosphoenolpyruvate
PPDK	pyruvate, phosphate dikinase
PP_i	inorganic pyrophosphate
PEPCK	phosphoenolpyruvate carboxykinase
PPases	pyrophosphatases
Ru5P	ribulose-5-phosphate
S7P	sedoheptulose-7-phosphate
H^+-PPase	proton-translocating pyrophosphatase

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6.1 Pyrophosphate-Dependent Phosphofructokinase and Methane Metabolism

Inorganic pyrophosphate (PP_i) is a by-product of numerous anabolic reactions, including the biosynthesis of nucleic acids, proteins, coenzymes, isoprenoids, and oligo- and polysaccharides. It has been proposed that the removal of PP_i by pyrophosphatases (PPases) makes biosynthetic reactions thermodynamically favorable (Kornberg 1962). An increasing body of biochemical and genetic evidences suggest that energy-rich PPi molecules also play an important role in the energy metabolism of many organisms (Gest 1972; Mansurova 1989; Baykov et al. 2013). Thus, in some organisms, an energy-consuming reaction of glycolysis—phosphorylation of fructose-6-phosphate (F6P) to fructose-1,6-bisphosphate (FBP)—is catalyzed by PP_i-dependent phosphofructokinase (PP_i-PFK, EC 2.7.1.90) instead of ATP-dependent phosphofructokinase (ATP-PFK, EC 2.7.1.11). In contrast to the ATP-PFK, PP_i-PFK catalyzes a reversible reaction:

$$\text{Fructose} - 6\text{P} + \text{PP}_\text{i} \overset{\text{Mg}^{2+}}{\rightleftarrows} \text{Fructose} - 1,6\text{P}_2 + \text{P}_\text{iT}$$

$$\text{Fructose-6P} + \text{ATP} \overset{\text{Mg}^{2+}}{\rightarrow} \text{Fructose-1,6P}_2 + \text{ADP}$$

ATP- and PP_i-PFKs share a common origin, but their evolutionary history is highly complex and includes "rampant" horizontal gene transfers. The specificity for PPi as a phosphoryl donor can be determined by two conservative amino acids, K104 and D124, in the active site of PP_i-PFK (relative to amino acid sequence of ATP-PFK from *Escherichia coli*), whereas ATP-PFKs possess either G104 and G124 or G104 and D124 (Chi and Kemp 2000). Indeed, mutation of just a few amino acids can change the substrate preference from PP_i to ATP (Chi and Kemp 2000). Phylogenetic analyses of ATP- and PP_i-PFKs show that changes between the phosphate donors in PFKs occurred more than once (Bapteste et al. 2003; Meurice et al. 2004).

The modified version of glycolysis where PP_i-PFK catalyzes the interconversion between F6P and FBP has been found in plants and many, predominantly anaerobic, microorganisms (Reeves et al. 1974, 1976; Mertens 1991; O'Brien et al. 1975). Surprisingly, PP_i-PFKs have been found in all aerobic methanotrophic *Gammaproteobacteria* and many *Alphaproteobacteria* (Khmelenina et al. 2011). The catalytic properties of the enzyme have recently been characterized and correlated with the C_1-assimilation pathways and aerobic metabolism (Beschastny et al. 1992; Reshetnikov et al. 2008; Rozova et al. 2010, 2012). Like other bacterial PP_i-PFKs, methanotrophic enzymes catalyze reversible reactions with almost equal specific activities in both directions and demonstrate higher affinities to FBP than to F6P. The higher efficiency of the enzymes with FBP as a substrate implies that the methanotrophic PP_i-PFKs might also be involved in gluconeogenesis. The capability of PP_i-PFK from methylotrophic bacteria with the Calvin cycle to catalyze phosphorylation of sedoheptulose-7-phosphate (S7P) suggests diverse functions of the

enzyme in carbon assimilation. Surprisingly, however, the thorough investigation of PFKs as well as other PP_i-dependent enzymes in methanotrophy uncovered more complex metabolic interactions between PP_i-pools, biosynthesis, and cell energetics.

6.2 Distribution of PP_i-PFKs in Methanotrophs and Methylotrophs

Analysis of bacterial genomes shows that genes homologous to the PP_i-PFK gene (*pfp)* are present in all methanotrophs of the families *Methylococcaceae* (*Gammaproteobacteria*) and *Methylocystaceae* (*Alphaproteobacteria*). However, *pfp*-like sequences are absent in facultative acidophilic methanotrophs belonging to the genera *Methylocella*, *Methylocapsa* and *Methyloferula* (family *Beijerinckiaceae*, class *Alphaproteobacteria*), and *Methylacidiphilum* (phylum *Verrucomicrobia*) (Table 6.1).

It is tempting to speculate that the distribution of PP_i-PFK could be related with peculiarities of the energy generation mechanisms in methane-oxidizing bacteria, specifically with those possessing the particulate methane monooxygenase (pMMO, a membrane-bound methane monooxygenase). In addition to methane oxidation, membranes of methanotrophs contribute to electron transfer and ATP synthesis via oxidative phosphorylation. Thus, methane oxidation and respiration processes occur simultaneously, suggesting complicated regulation. If there is an imbalance between their formation and reutilization, for example, during substrate limitation, oxygen radicals can disturb membrane integrity and hamper aerobic ATP synthesis (Murrell et al. 2000). As a consequence, the use of PP_i instead of ATP as a phosphoryl donor in some key metabolic reactions may provide a solution for these types of bioenergetic problems. In some instances it supports ATP synthesis via proton-translocating pyrophosphatase (H^+-PPase) thus bypassing the respiratory chain. The presence of *pfp* and the H^+-PPase gene (*hpp*) in genomes of autotrophic bacteria which oxidize NH^+_4 by ammonia monooxygenase (AMO), which is structurally and functionally similar to pMMO, additionally supports this hypothesis. Moreover, in chromosomes of *Nitrosomonas europaea*, *Nitrosospira multiformis*, and *Thiobacillus denitrificans*, *pfp* and *hpp* genes are colocated, further supporting the analogous functional implication. This is in accordance with the earlier suggestion that PP_i-PFK is restricted to organisms constrained in cell energy (Mertens 1991; Lopez-Marques et al. 2004).

It should be mentioned that among methanotrophs sequenced, only psychrophilic or psychrotolerant methanotrophs (*Methylomonas* spp., *Methylobacter tundripaludum*, and *Methylovulum miyakonense*) possess both ATP-PFK and PP_i-PFK. Thus, the functional role of both enzymes should be further investigated, specifically with respect to low-temperature methane utilization.

Among methylotrophs unable to grow under methane, only a few bacteria possess *pfps*: *Amycolatopsis methanolica*, *Methylibium petroleiphilum*, and all species of the *Methyloversatilis* genus as well as several representatives of the

Table 6.1 Distribution of PPi-dependent and PPi-related enzymes in aerobic methanotrophs

Methanotrophs	*pfp (pfk)*[1]	*hpp*	*fbp*	*ppdk (peps)*[2]	*pppc*
Gammaproteobacteria					
Methylobacter sp. BBA5.1	JQKS01_v1_50092[a]	JQKS01_v1_50091[a]	JQKS01_v1_150112	JQKS01_v1_40064	–
Methylobacter luteus	MLUTv2_130136[a]	MLUTv2_130135[a]	MLUTv2_10929	MLUTv2_10577, MLUTv2_60079, (MLUTv2_11498)	–
Methylobacter marinus	MMARv2_40152[a]	MMARv2_40151[a]	MMARv2_90235	MMARv2_10622, (MMARv2_80180)	–
Methylobacter tundripaludum	MTUNv2_10037 (MTUNv2_31312)	WP_040579994	MTUNv2_10036	(MTUNv2_10444)	MTUNv2_31234
Methylobacter whittenburyi	2585411845[a]	2585411844[a], 2585415787	2585413034	2585414876, (2585415392)	–
Methylocaldum szegediense	MSZE_100020[a]	MSZE_100021[a]	MSZE_160045	MSZE_35001, (MSZE_630019)	–
Methylocaldum sp. 175	2595101800	–	2595102746	2595103395	WP_085215389
Methylococcus capsulatus	AAU92465[a]	AAU92464[a], AAU92742	–	AAU90936	–
Methylogaea oryzae	2677967367	–	2677966511	2595103395	2677969795
Methyloglobulus morosus	AYLOv1_510051	AYLOv1_240003	AYLOv1_960008	AYLOv1_170016, (AYLOv1_600016)	AYLOv1_1140004
Methylohalobius crimeensis	WP_022948636	WP_022947739, WP_022949106	WP_022949028	(WP_022949066)	–
Methylomicrobium agile	WP_005370363	WP_031431143	WP_031429978	WP_031431086	WP_031430491
Methylomicrobium album	EIC28912	EIC28925	EIC31185	EIC28860, (EIC29443)	WP_040578267

Methylomicrobium alcaliphilum	CCE21914	CCE21914	CCE24525	CCE23390, (CCE23522)	WP_014148666
Methylomicrobium buryatense	WP_017840709	WP_017841210	WP_017840017	WP_017840279, (WP_017838940)	WP_017839449
Methylomicrobium kenyense	2590635905	2590635188	2590634304	2590636946, (2590636630)	2590633526
Methylomarinum vadi	WP_031435725	WP_031432221	WP_031433046	WP_031433822, (WP_031432428)	WP_031433131
Methylomonas denitrificans	**AMK76130**	**AMK77102**	**AMK79190**	**AMK78269, (AMK78203)**	JTDD01_v1_1250011
Methylomonas methanica	AEF99237	AEG00770	AEF99731	AEG00604, (AEG01450)	Metme_3540
Methylomonas lenta	WP_066981631	WP_066981622	WP_066978181	WP_066982808, (WP_066988046)	WP_066977012
Methylomonas koyamae	WP_064023958	–	WP_064028194	WP_064041892, (WP_064024278)	WP_064039842
Methylomonas sp. 11b	WP_036277424	–	WP_026600830	WP_026601247, (WP_026601308)	WP_026602146
Methylomonas sp. MK1	WP_026147146	–	WP_020481269	WP_020482381, (WP_020482453)	WP_020484767
Methylosarcina fibrata	WP_026223761	WP_026223692	WP_020562938	WP_020564235 (WP_020565392)	–
Methylosarcina lacus	WP_024298437	WP_084480070	WP_024298747	WP_024300064, (WP_02429660)	–
Methylovulum miyakonense	WP_019866931 (WP_040572823)	–	WP_019868142	(WP_019865768)	–

(continued)

Table 6.1 (continued)

Methanotrophs	*pfp (pfk)*[1]	*hpp*	*fbp*	*ppdk (peps)*[2]	*pppc*
Alphaproteobacteria					
Methylosinus trichosporium	2507407687	2507407023	2507406776	2507410009, (2507406191)	–
Methylosinus sp. LW4	WP_018266557	WP_026191235	WP_018266021	WP_018264986	
Methylocystis parvus	WP_016919815	WP_016919059	WP_016919097	WP_016920059, (WP_016920475)	–
Methylocystis sp. ATCC *49242*	WP_036288085	WP_036287353	WP_036282249	WP_036282642, (WP_036285759)	–
Methylocystis rosea	WP_018408941	WP_026223002	WP_018408205	WP_026222558, (WP_018409177)	–
Methylocystis sp. SB2	WP_029648267	WP_029651352	WP_029651487	WP_029650668, (WP_029648550)	–
Methylocystis sp. SC2	WP_014890254	WP_041927101	WP_014889587	WP_041927179, (WP_014890466)	–
Methylocapsa acidiphila	–	WP_026606263	WP_026605803	WP_026606112	–
Methylocapsa aurea	–	WP_036257977	WP_036264693	WP_036257747 (WP_036264495)	–
Methylocapsa palsarum	–	SFK27575	SFK74382	SFK10404	–
Methylocella silvestris	–	WP_012589699	WP_012590230	WP_012589757, WP_012592406	–
Methyloferula stellata	–	WP_026595767	WP_020174127	WP_020176621	–

Verrucomicrobia					
Methylacidiphilum infernorum	(ACD84176)	ACD84264	ACD83413	(ACD83642, ACD82080)	–
Methylacidiphilum fumariolicum	(CCG93087)	CCG93130	CCG91987, CCG91503	(CCG92186, CCG93310)	–
Methylacidiphilum kamchatkense	(JQNX01_v1_100047)	JQNX01_v1_40005	JQNX01_v1_20306, JQNX01_v1_10325	(JQNX01_v1_40201)	–

[a]*pfp* and *hpp* genes are colocated; *1*, number of gene coding for ATP-dependent 6-phosphofructokinase is given in the brackets; *2*, number of gene coding for PEP synthase is given in the brackets. *pppc*—gene for PPi-type PEP carboxykinase. The data were obtained from databases www.genoscope.cns.fr, http://www.ncbi.nlm.nih.gov/, and https://img.jgi.doe.gov

genus *Methylobacterium* (*Methylobacterium* sp. 4-46, *Methylobacterium* sp. WSM2598, *M. platani*, *M. aquaticum*, *M. tarhaniae*, *M. variabile*, and *M. nodulans*). The functional significance of PPi-PFK in non-methanotrophic methylotrophs is not clear. For the facultative methylotroph, *M. nodulans* ORS 2060, a relationship between PP_i-PFK and its phytosymbiotic lifestyle could be proposed. This bacterium is a rhizospheric phytosymbiont and diazotroph whose life cycle includes an anaerobic stage of bacteroids, during which it consumes products of plant photosynthesis and fixes atmospheric nitrogen in the legume tubercles (Renier et al. 2011). The anaerobic stage and N_2 fixation requires a mechanism for ATP acquisition from polycarbon substrates via PPi-dependent glycolysis. During growth on L-arabinose, cells of *M. nodulans* ORS 2060 had higher PP_i-PFK activity in comparison to methanol-growing cells (Rozova et al. 2012). Phylogenetically, *M. nodulans* PP_i-PFK is most closely related to the enzymes from the non-methylotrophic phytosymbionts *Rhizobium leguminosarum*, *Sinorhizobium medicae*, and *Agrobacterium radiobacter*, also stimulating nodulation in higher plants (64–65% identity). Such distribution may suggest a vital role of the PPi-dependent enzymes in the association of bacteria with higher plants.

6.3 PP_i-PFK from *Alphaproteobacteria*: *Methylosinus trichosporium* OB3b and *Methylobacterium nodulans* ORS 2060

In the alphaproteobacterial methylotrophs where C_3 compounds are the first products of carbon assimilation via the serine cycle, the gluconeogenic function of PPi-PFK is obvious. As expected, PPi-PFKs from methanotroph *Ms. trichosporium* OB3b and methanol-utilizing *M. nodulans* ORS 2060 have a higher efficiency with FBP compared to F6P (Rozova et al. 2012). The homohexameric enzyme of *Ms. trichosporium* (270 kDa) also catalyzes the phosphorylation of sedoheptulose-7-phosphate (S7P) but with much lower affinity than F6P (Table 6.2). The functional implication of PP_i-PFK in the Calvin cycle rearrangement reactions is discussed for the *Mc. capsulatus* Bath (see below). Such a capacity of the enzyme could partially be explained from an evolutionary point of view. Silent elements of autotrophic metabolism were found in the genomes of both methylotrophs, i.e., *Ms. trichosporium* has the gene coding for phosphoribulokinase (ID 2507407232), whereas *Mb. nodulans* harbors the genes for both key Calvin cycle enzymes, ribulose-1,5-bisphosphate carboxylase (ACL58349 and ACL57391) and phosphoribulokinase (ACL55364). Based on genomic data and PPi-PFK properties, one could speculate that the ancestor of these alphaproteobacterial methylotrophs was able to assimilate CO_2 and later gained the ability to use more reduced C_1 substrates via the serine cycle. This suggestion is in accordance with the hypothesis that the serine cycle must have evolved independently in various lineages of autotrophic *Alphaproteobacteria* (Beck et al. 2015).

Importantly, the activity of *Ms. trichosporium* PP_i-PFK with F6P or FBP is inhibited by ADP and AMP, and this inhibitory effect is more pronounced in the

Table 6.2 Some properties of methylotrophic PPi-PFKs

Parameters	*Methylomicrobium alcaliphilum* (Rozova et al. 2010)	*Methylomonas methanica* (Beschastny et al. 1992)	*Methylococcus capsulatus* (Reshetnikov et al. 2008)	*Methylosinus trichosporium* (Rozova et al. 2012)	*Methylobacterium nodulans* (Rozova et al. 2012)	*Amycolatopsis methanolica* (Alves et al. 1994)
V_{max}, U/mg						
Fructose-6P	577	840	7.6	88	86.89	107
Fructose-1,6P_2	805	850	7.6	89	74.7	n.d.
Sedoheptulose-7P	0.18	n.d.	31	85	46.56	n.d.
K_m, mM						
PPi	0.118	0.051	0.027	0.011	0.006	0.2
Fructose-6P	0.64	0.39	2.27	0.24	0.65	0.4
Sedoheptulose-7P	1.01	n.d.	0.03	0.51	0.21	n.d.
Pi	3.4	1.7	8.69	2.4	1.04	0.84
Fructose-1,6P_2	0.095	0.1	0.328	0.07	0.043	0.025
Mg^2+ (forward reaction)	0.22	0.038	0.028	0.030	0.047	0.04
Mg^2+ (reverse reaction)	0.33	0.35	2	0.363	0.32	0.77
*M*r, kDa (subunit number)	180 (4 × 45)	92 (2 × 45)	90 (2 × 45)	270 (6 × 45)	180 (4 × 45)	170 (4 × 45)
pH optimum	7.5	8.0	7.0	7.5	7.5	7.5
T optimum, °C	30	40	30	50	30	35–46
Effectors	No	No	No	AMP, ADP	No	No

n.d., not determined

gluconeogenic direction (Rozova et al. 2012). The influence of metabolites on PP_i-PFK activity has been registered only for the enzyme from the purple photosynthetic bacterium *Rhodospirillum rubrum* (Pfleiderer and Klemme, 1980). Obviously, the carbon flow from C_3 compounds to the biosynthesis of carbohydrates in *Ms. trichosporium* could be regulated at the level of PP_i-PFK activity as a first response to the overall energy balance.

6.4 PPi-PFKs from *Gammaproteobacteria*: *Methylomonas methanica* 12 and *Methylomicrobium alcaliphilum* 20Z

PP_i-PFKs from *Methylomonas methanica* 12 and *Methylomicrobium alcaliphilum* 20Z were found to be homodimeric (2 × 45 kDa) and tetrameric (4 × 45 kDa), respectively. Like other bacterial PP_i-PFKs, both enzymes catalyze the reversible reactions with almost equal specific activities in both directions, demonstrate higher affinities for fructose-1,6-bisphosphate (FBP) than to fructose-6-phosphate (F6P), and have the absence of allosteric effectors (Table 6.2). Higher efficiency with FBP as a substrate implies that the enzyme is involved predominantly in gluconeogenesis. Since both methanotrophs can cleave hexose phosphates into pyruvate and glyceraldehyde-3-phosphate via the nonreversible Entner-Doudoroff pathway (ED pathway), the participation of PP_i-PFKs in the equilibration of hexose phosphate and triose phosphate pools cannot be excluded in these bacteria. However, the gluconeogenic function of PP_i-PFK seems to be somewhat dispensable, as both microbes possess a fructose-1,6-bisphosphatase (FBPase), which irreversibly dephosphorylates fructose-1,6-bisphosphate onto fructose-6-phosphate. Furthermore, some gammaproteobacterial methanotrophs do not have ED genes. It has also been speculated that PPi-PFK contributes to recycle of PPi formed during the synthesis of sucrose and glycogen (Figs. 6.1 and 6.2), thus supporting carbon flow toward di- and polysaccharide biosynthesis.

The systems-level investigation of carbon flow in *Mm. alcaliphilum* 20Z highlighted the importance of PP_i-PFK in central metabolic pathways, as one of the key steps in carbon assimilation (Table 6.3, Kalyuzhnaya et al. 2013; Akberdin et al. 2018). With this pathway, the energy of PPi, a waste product of anabolic reactions, such as the synthesis of lipids, carbohydrates, proteins, and nucleic acids, can be reutilized. Reutilization of PP_i in the PP_i-mediated glycolytic pathway significantly increases the predicted efficiency of one-carbon assimilation. It has been also suggested that PPi-PFK is one of the key regulatory steps in cell growth (Akberdin et al. 2018). Since methane-derived formaldehyde is mostly oxidized via oxidative pentose phosphate pathways and intracellular PP_i mostly comes from biosynthetic reactions, the decision between C_1-oxidation and C_1-asimilation is made at the level of F6P. Upon active biosynthesis, cells have a sufficient supply of PPi, further supporting carbon assimilation by directing carbon toward pyruvate (i.e., F6P will be converted to FBP). However, if biosynthesis slows (e.g., cells experience NAD(P)H or ATP limitation), the supply of PP_i will drop, reducing carbon flow via PP_i-PFK. As a result, F6P will be converted to glucose-6-phosphate

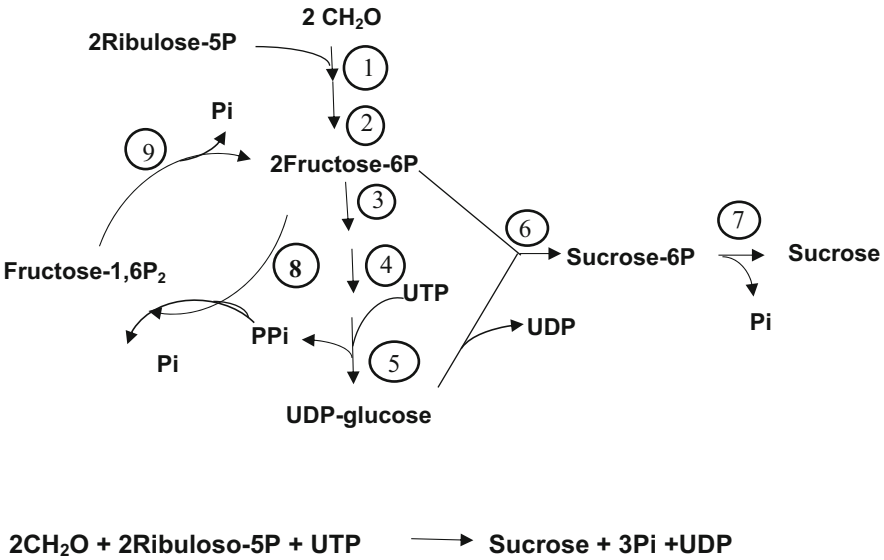

Fig. 6.1 The role of futile cycle in the PPi decay at sucrose synthesis by *M. alcaliphilum* 20Z. 1, hexulose-6-phosphate synthase; 2, hexulose-6-phosphate isomerase; 3, phosphoglucose isomerase; 4, phosphoglucomutase; 5, UDP-glucose pyrophosphorylase; 6, sucrose phosphate synthase; 7, sucrose phosphate phosphatase; 8, PPi-dependent 6-phosphofructokinase; 9, fructose-1,6-bisphosphatase

and shuttled into the oxidative pentose phosphate pathways for oxidation and restoration of redox and/or the ATP supply.

Finally, from a catalytic standpoint, the conversion of formaldehyde into pyruvate via glycolysis provides the rationale for the operation of methanotrophy in a fermentation mode and could explain distribution of methanotrophs in ecosystems with limited supply of oxygen (Kalyuzhnaya et al. 2013).

6.5 Multifunctional PPi-PFK from *Methylococcus capsulatus* Bath

Methylococcus capsulatus Bath is a somewhat unique gammaproteobacterial methanotroph as it possesses and expresses three different pathways for C_1 carbon assimilation: the RuMP pathway (assimilation of formaldehyde), the serine pathway (assimilation of formate), and the Calvin cycle (CO_2 fixation). PP_i-PFK from *Mc. capsulatus* Bath has been purified and shown to have very intriguing properties that are uncommon for other PPi-PFKs: along with F6P, the enzyme can phosphorylate S7P and ribulose-5-phosphate (Ru5P). The specific activity with S7P and affinity for this substrate are much higher (V_{max} = 31 U/mg of protein, K_m = 0.027 mM) than for F6P (V_{max} = 7.6 U/mg of protein, K_m = 2.27 mM); therefore the enzyme can be

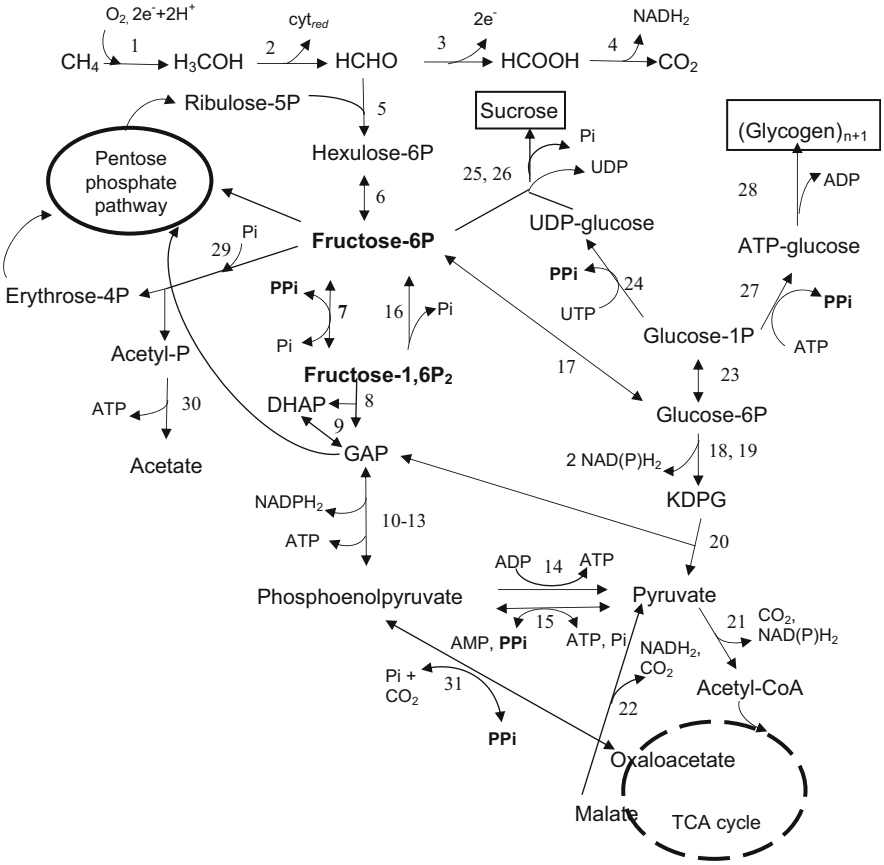

Fig. 6.2 Central carbon metabolism of *M. alcaliphilum* 20Z (modified from Rozova et al. 2010). 1, methane monooxygenase; 2, methanol dehydrogenase; 3, formaldehyde dehydrogenase; 4, formate dehydrogenase; 5, 3-hexulose-6-phosphate synthase; 6, 3-hexulose-6-phosphate isomerase; 7, PPi-dependent 6-phosphofructokinase; 8, fructose-1,6-phosphate aldolase; 9, triose phosphate isomerase; 10, glyceraldehyde 3-phosphate dehydrogenase; 11, phosphoglycerate kinase; 12, phosphoglycerate mutase; 13, enolase; 14, pyruvate kinase; 15, pyruvate, phosphate dikinase; 16, fructose-1,6-bisphosphatase; 17, phosphoglucose isomerase; 18, glucose-6-phosphate dehydrogenase; 19, 6-phosphogluconate dehydrogenase; 20, 2-keto-3-deoxy-6-phosphogluconate aldolase; 21, pyruvate dehydrogenase complex; 22, malic enzyme; 23, phosphoglucomutase; 24, UDP-glucose pyrophosphorylase; 25, sucrose phosphate synthase; 26, sucrose phosphate phosphatase; 27, -ADP-glucose pyrophosphorylase; 28, glycogen synthase; 29, fructose-6-phosphate/xylulose-5-phosphate phosphoketolase; 30, acetate kinase; 31, PPi-type phosphoenolpyruvate carboxykinase

designated as sedoheptulose-7-phosphate kinase (Reshetnikov et al. 2008). Hence, in the *Mc. capsulatus* Bath, the enzyme participates in rearrangement reactions of the RuMP and ribulose-1,5-bisphosphate (RuBP) pathways providing Ru5P or RuBP which are acceptors for formaldehyde or CO_2, respectively. The ability to phosphorylate Ru5P seems to be redundant in *Mc. capsulatus* Bath since the methanotroph

Table 6.3 Experimental data and in silico predictions of growth and O_2 consumption rates for *Mm. alcaliphilum* 20Z[a]

Metabolic step (Pathway/ enzyme)	Growth rate (h^{-1})	O_2 consumption rate (mmol g CDW $^{-1}$ h^{-1})
Experimental data	0.12 ± 0.01[b]	14.7 ± 0.9[b]
F6P → FBP (EMP/via PPi-PFK)	0.1499	13.82
F6P → FBP (EMP/via ATP)	0.1439	14.2
FBP → F6P (EDD/PPi-PFK)	0.1360	14.7
FBP → F6F (EDD/FBPase)	0.1360	14.7
Without H^+-PPase	0.1477	13.95
PEP → Oxaloacetate (via PPi–PEPC)	0.1504	13.79
Pyruvate → PEP (via PPDK)	0.1540	13.55

[a]The details of the flux balance model are described by Akberdin et al. (2018), summarized in Chap. 7; methane uptake set at 11.7 mmol g CDW^{-1} h^{-1}
[b]Continuous culture parameters (the growth rate represents 85% of μ_{max})

also has an active ATP-dependent phosphoribulokinase (Baxter et al. 2002). Such a metabolic redundancy has been connected to relatively high growth temperature of the microbe (Eshinimaev et al. 2004). A number of alternative metabolic roles have been proposed, including balancing F6P and FBP levels and contributing to energy metabolism (Reshetnikov et al. 2008, Khmelenina et al. 2011).

6.6 PP_i Metabolism in Methanotrophs

Among the other key PP_i-linked enzymes contributing to methanotrophy are pyruvate phosphate dikinase (PPDK), PPases, and phosphoenolpyruvate carboxykinase (PEPCK). PPDK catalyzes the reversible conversion of phosphoenolpyruvate (PEP) into pyruvate accompanied by ATP synthesis from AMP. With this enzyme, the glycolytic pathway became fully reversible and energetically 2.5 times more efficient in comparison to the classical glycolytic pathway (Mertens 1993; Slamovits and Keeling 2006). Unlike most microorganisms commonly disrupting PPi by soluble PPase, methanotrophs possess only a very low activity of the enzyme (Beschastny et al. 2008). However, many methanotrophs possess a proton-translocating V-type H^+-PPase (*hpp*) (Reshetnikov et al. 2008; Khmelenina et al. 2011). H^+-PPases represent membrane-bound, proton-translocating PPases which might contribute to regeneration of PPi from ATP. The predicted ratio of ATP hydrolysis: PPi formation for this class of enzymes is 1:3 (Scöcke and Schink 1998). Furthermore, the enzyme is often colocated and co-transcribed with the PPi-PFK gene. In *Mc. capsulatus* Bath, transcription of the *pfp-hpp* operon is initiated from a single σ^{70}-like promoter (initiation transcription site -10 (TAAGTT) and -35 (TTGTAA)) (Reshetnikov et al. 2008; Khmelenina et al. 2011). So, PP_i formed during dephosphorylation of FBP or S7P by PP_i-PFK can be used by H^+-PPase, generating an electrochemical proton gradient useful in cell bioenergetics.

The activity of the enzyme remains to be measured; however it is expected that the enzymes contribute to 25% of PP_i pool upon active growth on methane (Table 6.3, Akberdin et al. 2018).

The recent discovery of the gene coding for a putative phosphoenolpyruvate carboxykinase (PEPCK) fixing CO_2 to PEP to produce oxaloacetate and PP_i or vice versa (Chiba et al. 2015) showed additional redundancy of the enzymes involved in glycolysis/gluconeogenesis in some methanotrophs (Table 6.1). Because PEP is a key intermediate in many metabolic processes, PEPCK may work as a major crossroad that connects glycolysis/gluconeogenesis and organic acid metabolism like the tricarboxylic acid cycle. Confirmation of functionality of the third PP_i-dependent enzyme of glycolysis/gluconeogenesis will help us to understand the central metabolism of methanotrophs more deeply.

The role of the PP_i-linked enzymes is exemplified on *M. alcaliphilum* (Fig. 6.2). A simple flux balance analysis highlighted the efficiency of PP_i-dependent reactions (Table 6.3). The results support the scenario in which PPi-dependent reactions improve overall efficiency of methane utilization, by allowing a larger portion of methane to be assimilated. Thus the replacement of key metabolic steps with PP_I-driven reactions might represent a vital adaptation upon carbon and/or oxygen limitation.

Acknowledgments The work was supported by the Russian Foundation for Basic Research #18-04-00771 and by the US Department of Energy Bioenergy Technologies Office (DOE-BETO under contract No. DE-AC36-08GO28308).

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Systems Biology and Metabolic Modeling of C_1-Metabolism

7

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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

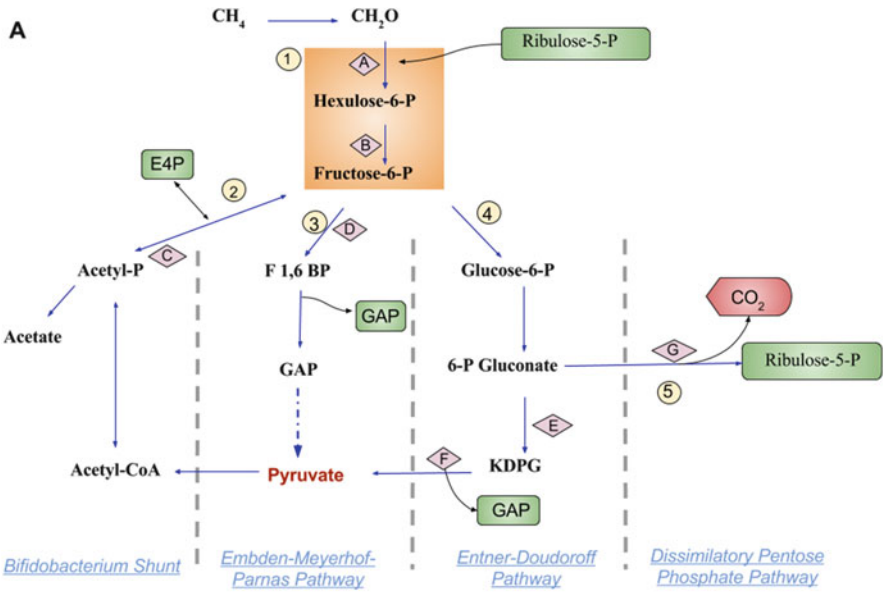
Table 7.1 Overview of system-level investigation of methanotrophy

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

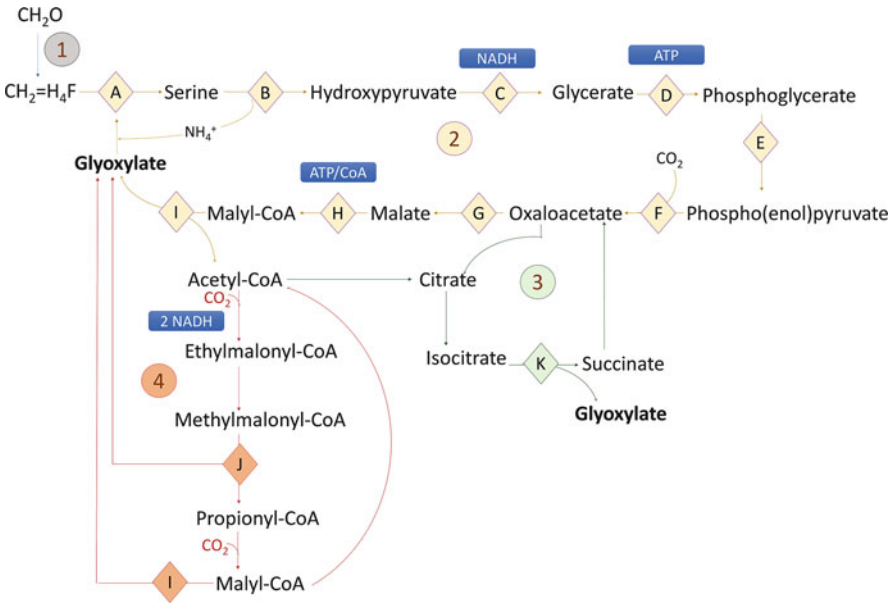


Fig. 7.2 Serine cycle and its variants. (1) Three various pathways have been proposed for methylenetetrahydrofolate formation, including spontaneous condensation with tetrahydrofolate, condensation facilitated by a formaldehyde-activating enzyme (Fae), and tetrahydrofolate-linked C_1-transfer; (2) the 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.

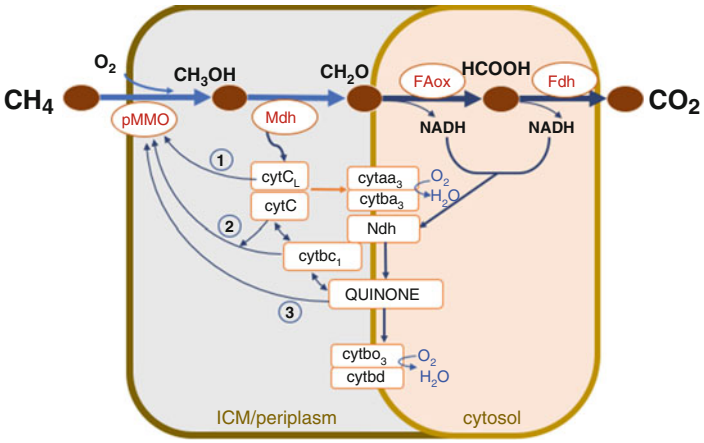


Fig. 7.3 Electron transfer reactions in *Methylomicrobium alcaliphilum* 20Z. 1–3 represent different pathways for redox supply for pMMO reaction: (1) direct coupling mode, (2) uphill electron transfer mode, and (3) redox mode. M. *alcaliphilum* 20Z has various terminal cytochrome oxidases, whose expressions 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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Metabolic Engineering of Methanotrophic Bacteria for Industrial Biomanufacturing

8

Calvin A. Henard and Michael T. Guarnieri

8.1 Introduction

The advent of next-generation sequencing technology, systems-level analyses capabilities, and synthetic biology tool development has enabled rapid advances in microbial metabolic engineering. Hypothesis-driven, targeted strain-engineering approaches have now been deployed in an array of microbes, facilitating elucidation of fundamental mechanisms governing microbial metabolism and metabolic networks and leading to the generation of biocatalysts with diverse functionality and product suites. This has included development of microbes with enhanced substrate utilization capacity and rate, novel biosynthetic capacity, enhanced productivity metrics, and improved end-product tolerance (Woolston et al. 2013). Such advances have impacted industrial manufacturing, with applications ranging from bioenergy to biomedicine, enabling microbial production of "green" fuel and chemical intermediates, biopolymers, nutraceuticals, pharmaceuticals, and beyond (Clomburg et al. 2017).

Though significant advances have been made in conventional model and industrial microbes (i.e., *Escherichia coli* and *Saccharomyces cerevisiae*), fewer have been made in non-model heterotrophs, and fewer still have been realized in methanotrophic bacteria. Such advances have conventionally been limited by the absence of genetically tractable non-model organisms and/or lack of facile, efficient genetic engineering tools. However, a series of recent advances in methanotrophic genetic tool development have opened the door for targeted metabolic engineering strategies, including the identification of broad host-range plasmid systems (Ojala et al. 2011; Puri et al. 2015), inducible promoters (Henard et al. 2016), and, importantly, novel strains with characteristics well suited for genetic manipulation (e.g., relatively rapid growth rates and robust lab cultivation capacity) (Kalyuzhnaya

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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_8

et al. 2013; Gilman et al. 2015). These strains and tools, combined with a rapidly increasing understanding of fundamental methanotrophic metabolism, present the opportunity to implement an array of rational metabolic engineering strategies in these promising production hosts.

In this chapter, we review historic and recent advances in aerobic methanotrophic metabolic engineering, including progress in methanotrophic genomics and genetic tool development and implementation. We discuss methanotrophic metabolic diversity and the potential for utilization of methanotrophs as biocatalysts for the production of fuels, chemicals, and high-value products, including key techno-economic drivers and deployment limitations related thereto. Lastly, we explore future engineering strategies aimed at overcoming these bioprocess hurdles, specifically targeting enhancement of methane (CH_4) utilization, carbon- and energy-efficient biocatalysis, and complete utilization of renewable biogas sources. Such strategies offer a path forward to bring to bear the full potential of methanotrophic biocatalysis.

8.2 Methanotroph Genetics and Tools

8.2.1 Genomic and Functional Omic Analyses

The Organization of Methanotroph Genome Analysis (OMeGA) has recently sequenced and annotated multiple methanotroph genomes (Boden et al. 2011; Khmelenina et al. 2013; Flynn et al. 2016; Hamilton et al. 2015), adding to the available genome sequences for an array of methanotrophic bacterial genera (see www.methanotroph.org, Table 5.1). Genomic analysis of these methanotrophic bacteria indicates that significant divergence between closely related species exists, but core C1 metabolic pathways are conserved across diverse genera. These sequences have in turn led to unprecedented insight into the genetic blueprints of methanotrophs and guided genome-scale biochemical network reconstructions and preliminary metabolic flux models (la Torre de et al. 2015; Akberdin et al. 2018). Additionally, other systems-level transcriptomic and metabolomics studies are providing further insight into the central metabolic pathways required for CH_4 utilization in alphaproteobacterial (Matsen et al. 2013; Kalyuzhanaya et al. 2013; Vorobev et al. 2014) and gammaproteobacterial methanotrophs (Kalyuzhnaya et al. 2013; la Torre de et al. 2015). Recent transcriptomic profiling in *M. capsulatus* (Bath) suggests that significant alterations in energy metabolism occur during copper-replete or-deplete growth, corresponding to pMMO- or sMMO-dependent growth, respectively(Larsen and Karlsen 2016). These data also provide further evidence that quinones likely serve as the electron donors for pMMO-dependent CH4 oxidation (Larsen and Karlsen 2016), although definitive evidence for this is still lacking. Collectively, these datasets will assist in the identification of metabolic bottlenecks and carbon sinks and guide rational engineering of gene targets in these organisms.

8.2.2 Genetic Manipulation of Methanotrophic Bacteria

8.2.2.1 Metabolic Engineering Systems

Initial efforts to generate methanotrophic variants employed random mutagenesis approaches. Efforts to isolate antibiotic or amino acid analogue-resistant mutants in *Methylococcus* and *Methylomonas* using chemical (e.g., nitrosoguanidine, ethyl methanesulfonate) or ultraviolet light mutagenesis indicate that increasing spontaneous beneficial mutations in methanotrophs is difficult (Williams 1977; Harwood et al. 1972). These results also suggest that both *Methylococcus* spp. and *Methylomonas* spp. have high-fidelity DNA repair systems that may limit combinatorial metabolic engineering techniques (Williams and Bainbridge 1976). In contrast, methanotrophic strains capable of growth on CH_3OH, but not CH_4, have been isolated using dichloromethane (Nicolaidis and Sargent 1987; McPheat et al. 1987), which is converted to cytotoxic carbon monoxide by pMMO. Dichloromethane-resistant strains exhibited a loss of MMO activity, presumably due to inactivating mutations in pMMO, although DNA sequencing was never performed to verify alterations at the nucleotide level. Laboratory-evolved rifamycin-resistant methanotrophic strains are also readily obtained (Puri et al. 2015). Combined, these results suggest that spontaneous mutagenesis is not a limiting factor in obtaining mutant variants, but rather selective pressure and screening methodologies present a metabolic engineering hurdle. Further, these results indicate that improved strains can potentially be obtained through combinatorial metabolic engineering or directed evolution approaches in these organisms.

Transposon insertional mutagenesis offers a high-throughput approach to produce methanotroph mutant libraries and has successfully been employed to introduce mutations in these organisms. For example, a Tn5 system was leveraged to generate several *Methylomonas* spp. mutants deficient in nitrogen fixation (Toukdarian and Lidstrom 1984). Further, a similar system containing promoterless carotenoid genes was used to increase carotenoid production 20-fold compared to wild type in *Methylomonas* 16a (Sharpe et al. 2007). Such random approaches offer the potential for the generation of large genetically variable libraries and, in turn, isolation of optimal, process-relevant phenotypes.

Conventional genetic tools are relatively limited for use in methanotrophic bacteria compared to model organisms like *Escherichia coli*. However, both gene overexpression and gene disruption can be achieved in these organisms, allowing for the most basic rational metabolic engineering approaches. Incompatibility group P (IncP), incompatibility group Q (IncQ), and pBBR-based broad host-range plasmids have served as backbones to generate expression vectors for homologous/heterologous gene expression in methanotrophs. Successful expression of an array of heterologous antibiotic resistance genes and replicon-associated genes required for plasmid maintenance suggests that several promoters and ribosomal binding sites are recognized by these organisms. For example, both *E. coli* and methanotroph-derived promoters have been used for constitutive expression, and inducible promoters (such as the tetracycline promoter/operator) have been employed for fine-tuned, temporally regulated gene expression (Ojala et al. 2011; Puri et al. 2015; Henard et al.

2016; Kalyuzhnaya et al. 2015). Gene expression analyses during *Methylomicrobium alcaliphilum* growth on CH_4 have provided insights into promoter strength, which, not surprisingly, indicate that the pMMO operon (*pmoC*, *pmoA*, *pmoB*), methanol dehydrogenase (*mxa*), and hexulose phosphate isomerase (*hpi*) gene expression are robust (Kalyuzhnaya et al. 2013). We, and others, have utilized the methanol dehydrogenase *mxa* promoter (P_{mxa}) for high, constitutive gene expression in *Methylomicrobium* spp. (Puri et al. 2015; Henard et al. 2016).

Our group leveraged the available tools for use in methanotrophs to develop an inducible expression vector that enabled bioconversion of CH_4 to lactic acid, a precursor used to make the biodegradable plastic polylactic acid (PLA). Heterologous expression of a *Lactobacillus helveticus* L-lactate dehydrogenase in *M. buryatense* resulted in the production of 0.8 g/L lactic acid, the highest titer of any engineered target molecule derived from CH_4 to date (Henard et al. 2016). For future pathway engineering, it is worth noting that spatial constraints related to the intracytoplasmic membrane could pose issues with protein localization and function inside methanotrophs. Indeed, heterologous expression of reporters such as GFP, dTomato, and XylE functions more efficiently in whole-cell lysates compared to intact cells (Puri et al. 2015; Yan et al. 2016; Mustakhimov et al. 2016). Although it is unclear whether the internal membrane structure plays a role in the aforementioned phenotypes, spatial constraints imposed by the membrane could hinder multienzyme biosynthetic pathway functionality.

Broad host-range plasmids have also been employed to generate chromosomal integrants and knockout mutant strains. Genes can be inserted or deleted from the chromosome by standard homologous recombination methods using flanking regions of genomic sequence surrounding the target integration/gene site. Genetic knockout experiments using such methodology were successfully performed in the methylotroph *Methylobacterium extorquens* AM1 using the pCM184 vector (Marx and Lidstrom 2002). This vector contains a kanamycin antibiotic marker flanked by loxP sequences that enable removal and reuse of the marker via expression of the Cre recombinase. pCM184, or its derivatives, has also been used to successfully disrupt genes in methanotrophs *M. alcaliphilum* and *M. buryatense* (Puri et al. 2015). Thus, heterologous expression of the Cre recombination along with antibiotic resistance genes can be viewed as the first foreign genes to be expressed in a methanotrophic host.

Gene knockouts have since been generated in *M. buryatense* and *Methylobacter tundripaludum* by direct electroporation of a linear DNA fragment containing either an antibiotic cassette and a counterselection marker or an antibiotic marker flanked by flippase (Flp) recognition target (FRT) sites (Yan et al. 2016; Puri et al. 2016). In the latter case, removal of the antibiotic marker is achieved by heterologous expression of Flp. In both the loxP/Cre and FRT/Flp site-specific recombination systems, a scar is left on the chromosome that may present issues in industrial processes, wherein recombination at scar sites leads to deleterious removal of critical genetic components. Alternatively, unmarked mutants can be made using counterselection systems, such as sucrose counterselection. A sacB-based counterselection system has been successfully demonstrated in several methanotrophs, including

M. buryatense, *M. capsulatus*, *M. tundripaludum*, and *Methylomonas* (Ojala et al. 2011; Puri et al. 2015; Yan et al. 2016; Marx 2008).

8.2.2.2 DNA Delivery

Conjugation is the most widely used method for routine mobilization of plasmids, which is typically mediated by *E. coli* S17-1 via biparental mating. For detailed descriptions of conjugation methodologies in methanotrophs, refer to (Ojala et al. 2011; Puri et al. 2015; Kalyuzhnaya et al. 2015; Lee et al. 2016). Electroporation of methanotrophs, in contrast, has proven to be difficult with few research groups reporting successful, repeatable electroporation methodologies. Successful transfer of IncP-based plasmids to *Methylococcus capsulatus* via electroporation was initially reported over 25 years ago (Murrell 1992). Type II methanotrophs *Methylocella silvestris* BL2 *and Methylocystis* sp. strain SC2 have also been successfully transformed by electroporation (Baani and Liesack 2008; Crombie and Murrell 2011). However, routine electroporation methodologies following these reports were not established, underscoring the intrinsic difficulties in electroporation of these organisms. Thus, the methylotroph research community has actively focused on the development of electroporation methodologies for decades.

Recently, a promising study published by Yan and co-workers demonstrated successful electroporation of three type I gammaproteobacterial methanotrophs (Yan et al. 2016). In this report, *E. coli*-derived plasmids were unable to be transformed into *Methylomicrobium buryatense* via electroporation; however, plasmids isolated from methanotrophs that were originally transformed via conjugation were readily transformed. These data, along with genomic sequences, suggested that robust restriction systems encoded by these organisms recognize and cleave specific methylation patterns on DNA and have likely been responsible for the difficulty in routine electroporation of methanotrophs with *E. coli*-derived plasmids. In the report, several restriction systems were disrupted after electroporation of PCR-derived DNA, but removal of individual systems was unable to increase transformation efficiencies. Native restriction systems likely serve redundant roles to protect methanotrophs from invading foreign DNA; thus, iterative disruption of all restriction systems could improve transformation efficiencies and potentially allow expansion of the genetic toolbox for use in industrial methanotroph strain development.

8.2.3 Future Genetic Tool Development

A set of pBBR-based vectors previously generated for genome-wide library construction (Lynch and Gill 2006) could be easily altered for use in methanotrophs by swapping the *E. coli lac* promoter with methanotroph-specific promoters, assuming that the P_{lac} promoter isn't functional in a methanotroph. However, the *E. coli* P_{tac} promoter is recognized by *M. buryatense* (Puri et al. 2015); thus, these vectors may be suitable for direct use and library construction in this organism. Unfortunately, transformation efficiencies are quite low (typically 5×10^5 cfu/ug DNA delivered)

in the most industrially promising strains tested to date (Yan et al. 2016), which would limit library size.

In addition to the suite of broad host-range vectors currently available for use in methanotrophs, large native plasmids appear to be common in these organisms. Indeed, plasmids typically between 50K bp and 200K bp have been found in all organisms examined to date, with the exception of *M. capsulatus* (Lidstrom and Wopat 1984; Vuilleumier et al. 2012). The mechanism of replication of these plasmids is unknown, but their replicons could be used in the engineering of novel vectors for use in methanotrophs, which could have superior stability compared to the currently used IncP-based plasmids. Incompatibility of these native plasmids with plasmids housing heterologous replicons could be a potential reason for the limited number of origins that are stably replicated by methanotrophic bacteria.

Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated (CRISPR-Cas) systems are widespread in archaea and bacteria where they serve as an adaptive immune response to protect against foreign invaders (Makarova et al. 2015). These RNA-based systems have been co-opted as powerful tools for genome editing and transcriptional regulation in an array of heterologous hosts (Marraffini 2016; Komor et al. 2017; Mohanraju et al. 2016). With available expression vectors for use in methanotrophs, heterologous expression of CRISPR-Cas systems could revolutionize engineering in these biocatalysts. The type II, Cas9-based CRISPR system of *Streptococcus pyogenes* is the most characterized and has been successfully utilized for genome engineering in a variety of heterologous hosts (Marraffini 2016). However, toxicity related to Cas9 expression and off-target effects in some hosts have led to further research and expansion of the CRISPR repertoire. For example, other orthologous systems, like the type II–V Cpf1-based system of *Francisella*, offer versatility of CRISPR-based technology utilizing alternative endonucleases with differing recognition sites and CRISPR RNA maturation mechanisms (Zetsche et al. 2015; Ungerer and Pakrasi 2016). Further, several methanotrophs possess native CRISPR systems. Gene organization of the Cas array and the presence of a Cas3 endonuclease homolog suggest that the native CRISPR-Cas are type 1 systems similar to that in *E. coli* (Makarova et al. 2015). Importantly, to realize the full potential of these tools, transformation efficiencies need to be at least an order of magnitude greater than those currently achieved in methanotrophs (Yan et al. 2016). To this end, we are actively focused on improving transformation efficiencies in methanotrophic bacteria by leveraging bacteriophage lambda red recombineering (Sharan et al. 2009; Wang et al. 2009) and improving DNA delivery and incorporation methodologies.

8.3 Methanotrophic Metabolic Plasticity: Industrial Potential for CH_4 Biocatalysis

CH_4 offers a promising, high-volume petroleum replacement for fuel and chemical bioprocesses (Fig. 8.1) (Clomburg et al. 2017; Conrado and Gonzalez 2014; Haynes and Gonzalez 2014; Fei et al. 2014). Recent advances in gas recovery technologies

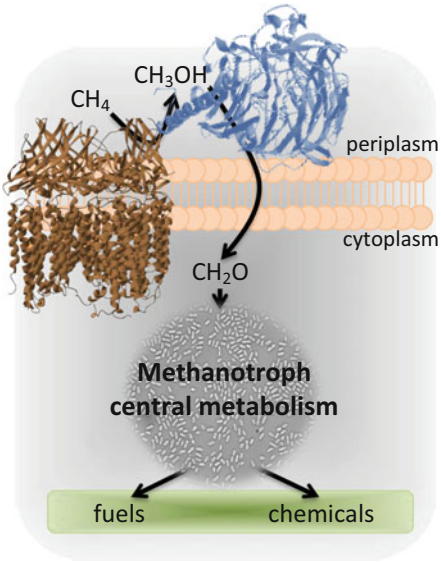

Fig. 8.1 Methane biocatalysis in aerobic gammaproteobacterial methanotrophs Methane (CH_4) is activated by the membrane-bound particulate methane monooxygenase to methanol (CH_3OH) in the periplasmic space, which is further oxidized to formaldehyde (CH_2O) by methanol dehydrogenase. CH_2O is assimilated via the ribulose monophosphate pathway in gammaproteobacterial methanotrophs Hexoses formed by the condensation of CH_2O and ribulose-5-phosphate enter the Embden-Meyerhof-Parnas pathway. Carbon flux through the pyruvate or acetyl-CoA hubs can be exploited by upgrading these versatile metabolites to a variety of fuel molecules and chemicals

have facilitated access to previously inaccessible natural gas reserves, while biogas generated from anaerobic digestion of waste streams offers a versatile, renewable CH_4 source. Importantly, CH_4 is also the second most abundant greenhouse gas (GHG), with nearly 60% of emissions derived from anthropogenic sources. However, the gaseous state of CH_4 makes for a lack of compatibility with current transportation and industrial manufacturing infrastructure, limiting its utilization as a transportation fuel and intermediate in biochemical processes. Resurgent interest in CH_4 upgrading has pushed microbial conversion of CH_4 to fuels and value-added chemicals to the forefront of industrial bioprocessing. CH_4 bioconversion offers both CH_4 valorization and GHG emission reduction potential and importantly offers a scalable, modular, and selective approach to CH_4 utilization compared to conventional physical and chemical conversion strategies. However, as noted above, advances in CH_4 biocatalysis have been constrained by limited genetic tractability of natural CH_4-consuming microbes. Additionally, these organisms often display low native productivity and conversion capacity.

Preliminary techno-economic analyses (TEA) by our group have identified key cost drivers in industrial deployment of methanotrophic biocatalysts, including carbon yield (g product/g CH_4) and volumetric productivity (g/L/h). These metrics dictate CAPEX requirements related to configuration and scale of a CH_4

bioconversion facility, including costs associated with CH_4 sourcing, the necessity for gas recycling and compression technologies, and bioreactor size and quantity. Thus, metabolic engineering strategies targeting enhancement to carbon conversion efficiency and productivity offer promising routes to improve process economics. TEA-driven metabolic engineering strategies may also inform selection of target products. For example, in conventional bubble column or continuous stirred-tank reactor configurations, there is the potential necessity for gas recycling to enable viable process economics. Such configurations may lend themselves to metabolic engineering pursuits targeting the production of gaseous products (such as volatile hydrocarbons), which could be readily isolated from off-gas streams prior to recycling. Alternatively, configurations that employ cell immobilization strategies may lend themselves to selection of excreted target products (e.g., organic acids). For extensive review of potential product suites from CH_4, please refer to (Kalyuzhnaya et al. 2015; Lee et al. 2016; Strong et al. 2015).

8.4 Future Rational Engineering Strategies

8.4.1 Increase CH_4 Assimilation Rate and Energy Efficiency

As noted above, CH_4 fermentation presents unique process hurdles, including gas mass transfer limitations. The rate of CH_4 mass transfer is in part a function of reactor configuration and ultimately defines the maximum potential CH_4 conversion rate, which in turn defines the maximum potential volumetric productivity in a methanotrophic biocatalyst. Efforts to enhance k_La are primarily restricted to the physical realm of fermentation engineering via optimization of reactor design (for detailed discussion of gas fermentation, please refer to Chap. 5). If sufficient k_La enhancement is achieved, the primary biological barrier to CH_4 utilization is rate of oxidation and assimilation. Thus, targeted improvements to monooxygenase (MMO) and downstream components of the CH_4 oxidation and assimilation machinery may enable enhanced rates of CH_4 utilization. Metabolic engineering strategies targeting a decrease in CO_2 evolution, expanded carbon utilization, and removal of competitive carbon pathways offer additional opportunities to enhance product yield.

CH_4 oxidation employs a unique suite of enzymes, including CH_4 monooxygenase and methanol dehydrogenase (MDH), which catalyze CH_4 conversion to methanol and methanol conversion to formaldehyde, respectively. Two forms of MMO have been identified in methanotrophic bacteria: particulate (pMMO) and soluble (sMMO). Both have been extensively biochemically and structurally characterized [for extensive reviews, please refer to (Lawton and Rosenzweig 2016; Sirajuddin and Rosenzweig 2015]. Similarly, MDH has been comprehensively characterized [for review, please refer to (Culpepper and Rosenzweig 2014; Anthony and Williams 2003)].

Limited progress has been made in the realm of pMMO engineering, for which the definitive mechanism of CH_4 oxidation, flux/rate, and reductant requirement

(s) remain elusive. However, pMMO enhancement via targeted protein engineering may enhance rates of CH_4 utilization and/or elucidate downstream bottlenecks. The system is encoded by the *pmoCAB* operon, organized into a α3β3γ3 trimer (Lawton and Rosenzweig 2016; Sirajuddin and Rosenzweig 2015). It has been indicated, although not experimentally confirmed, that the catalytic site is located in pmoB gene containing a dicopper center coordinated by three histidines. CRISPR-based strategies coupled to high-throughput oligo generation offer the unique opportunity to generate large pMMO libraries, with the potential to introduce thousands of combinations of mutations simultaneously (Garst et al. 2017). Such mutations could be targeted to *pmoB*, or alternatively, based upon preliminary structural information, targeted interfacial trimeric mutations. Similar strategies could also be concurrently employed in MDH to couple such targeted protein engineering strategies. However, given the potential that CH_4 oxidation does not represent the rate-limiting step in CH_4 utilization, metabolic engineering will ultimately need to be coupled with iterative flux balance analyses to elucidate putative downstream bottlenecks.

8.4.2 Maximize Carbon Conversion to Central Metabolites

8.4.2.1 KO Competitive Carbon Pathways

Gammaproteobacterial methanotrophs accumulate a substantial portion of their biomass as glycogen (40% g/g assimilated CH_4) and also secrete an extrapolymeric substance (30% g/g assimilated CH_4) that significantly decreases potential carbon flux to target product(s) (Gilman et al. 2015; Malashenko et al. 2001). Other molecules that can be viewed as either viable products or carbon sinks include excreted organic acids (acetate, succinate, lactate, formate) and sucrose and ectoine that are synthesized as osmoprotectants in halophilic methanotrophs (Lee et al. 2016; Strong et al. 2015).

The majority of viable fuel and high-value chemical intermediates are derived from either the primary pyruvate or acetyl-CoA metabolic nodes. Thus, increasing carbon flux to these nodes is a primary focus in ongoing engineering efforts. Iteratively, disrupting the aforementioned primary carbon sinks via gene knockout is a straightforward approach to potentially achieve increased flux to pyruvate and/or acetyl-CoA and prevent the pool of these key metabolites from conversion to unwanted side products. However, the importance of these carbon sink pathways in methanotroph physiology and their interrelationship with other intermediary metabolism remains to be fully elucidated. Thus, disruption of these potentially essential pathways could dramatically affect cell growth, redox balance, or long-term survival.

8.4.2.2 Bypass Decarboxylation Reactions

Recent research in the gammaproteobacterial methanotrophs indicate that these organisms preferentially flux carbon through a canonical Embden-Meyerhof-Parnas (EMP) pathway (Kalyuzhnaya et al. 2013), making conventional industrial strain-

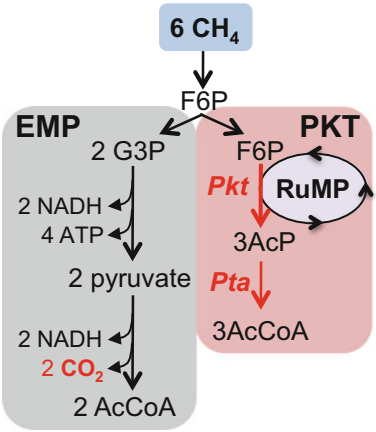

Fig. 8.2 Carbon-efficient biocatalysis using the phosphoketolase pathway. CH_4 is assimilated to fructose-6-phosphate (F6P) in gammaproteobacterial methanotrophs, which enters the Embden-Meyerhof-Parnas (EMP) pathway. F6P and/or xylulose-5-phosphate (X5P) can be converted to intermediates of the ribulose phosphate (RuMP) pathway and acetyl-P (AcP) by phosphoketolase (PKT, red arrows). AcP is readily converted to acetyl-CoA (AcCoA) by the phosphotransacetylase (Pta), bypassing the CO_2 lost in the decarboxylation of pyruvate to AcCoA (Adapted from (Henard et al. 2015)

engineering routes from sugars to biochemical intermediates and products possible in these CH_4-utilizing bacteria. A limitation for EMP pathway-based engineering is that one-carbon equivalent is lost in the decarboxylation of pyruvate to acetyl-CoA during aerobic catabolism, limiting theoretical acetyl-CoA-derived product yields. The phosphoketolase (PKT) pathway (also called the bifid shunt) represents an alternative route to acetyl-CoA that bypasses pyruvate decarboxylation. Engineering a heterologous phosphoketolase pathway has increased acetyl-CoA-derived product yields in an array of sugar-utilizing organisms (Henard et al. 2015). This approach can be mirrored in type I methanotrophs to generate stoichiometric amounts of acetyl-CoA from C1 substrates (Fig. 8.2). Of note, an obligate PKT-dependent pathway that bypasses downstream glycolytic reactions that produce reducing equivalents is redox neutral, which may limit cellular reducing power. This loss in reducing power could prove to be problematic if product biosynthesis is reducing equivalent demanding. In addition to being redox neutral, an obligate PKT pathway to acetyl-CoA does not generate ATP through substrate-level phosphorylation. However, it is unknown if methanotrophic growth is ATP and/or reducing power limited and to what extent, if any, EMP-derived ATP contributes to overall cellular energy. A model evaluating carbon flux through a PKT pathway vs. the EMP pathway indicates that an obligate PKT pathway would decrease biomass accumulation as more carbon is diverted through the dissimilatory pathway as a means to produce reducing power (la Torre de et al. 2015). However, recent results by our group have demonstrated overexpression of PKT isoforms enhance biomass yield from both CH_4 and methanol (CH_3OH), without reduction in lipid content,

suggesting that cellular reducing power is either not limiting or that reducing power is compensated for without reducing carbon flux to biomass (Henard et al. 2017).

Interestingly, several methanotrophic bacteria likely already utilize a PKT pathway for sugar phosphate catabolism. Genomic query of methylotrophs indicates that many of these organisms encode native PKTs (Rozova et al. 2015; Sánchez et al. 2010). This observation is intriguing since the majority of organisms that encode PKTs ferment hexoses and/or pentoses through this pathway under anaerobic conditions (Rohr et al. 2002). It seems unlikely that obligate methanotrophs ferment anaerobically because MMO requires equimolar oxygen to convert CH_4 to CH_3OH. However, recent data indicate that *Methylomicrobium* spp. switch to a fermentative-type metabolism under microaerobic conditions, excreting acetic acid, lactic acid, formic acid, succinic acid, and hydrogen gas (Kalyuzhnaya et al. 2013). The native PKT transcription was shown to increase under these microaerobic conditions, suggesting that this enzyme is likely involved in CH_4 fermentation. Thus, PKT engineering strategies may be more suitable for bioconversion of CH_4 to the above organic acids.

8.4.3 Complete Utilization of Biogas Carbon

Methanotrophs hold great promise for the capture and conversion of CH_4 from anaerobic digestion-derived biogas. However, much of the current research focus is on CH_4 upgrading, which is only one of the two major biogas components, with CO_2 typically comprising ~40% of biogas streams. The development of an industrially relevant biocatalyst capable of co-utilization and conversion of CO_2 and CH_4 would allow for complete utilization of biogas streams and, in turn, enhanced carbon conversion efficiencies. Additionally, development of a CH_4/CO_2 CO-utilizing biocatalyst would dramatically shift the landscape of greenhouse gas mitigation, capture, and conversion pursuits, providing a novel, photosynthesis-independent CO_2 biocatalyst.

Several proteobacterial methanotrophs encode the CO_2-fixation enzyme, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), ubiquitous in photoautotrophs. However, autotrophic metabolism has long been presumed to be absent in methanotrophs due to the significant ATP requirement associated with RuBisCO-dependent CO_2 fixation. Recent studies have challenged this dogma, demonstrating that some RuBisCO-encoding methanotrophs, such as *Methylococcus capsulatus*, can grow autotrophically if supplied with an energy source, although much slower compared to growth on other C1 substrates (Baxter et al. 2002). Further, recent evidence indicate that members of the NC10 phyla fix CO_2 (Khadem et al. 2011; Rasigraf et al. 2014) and *Verrucomicrobia* methanotrophs acquire 100% of their biomass from RuBisCO-assimilated CO_2, while CH_4 oxidation provides cellular energy in the form of ATP. CO_2 fixation has also been observed in type II methanotrophs that do not encode RuBisCO, presumably through carboxylation reactions in the serine cycle. Approximately 60% of biomass carbon in *Methylosinus trichosporium* OB3b is derived from CO_2 (Kalyuzhnaya 2013), but similar to

Verrucomicrobia, type II methanotrophs do not display optimal biocatalyst characteristics such as high growth rate and facile genetic tractability. Substantial overlap in CH_4 and CO_2 assimilation pathways suggests that an evolutionary link may exist between methanotrophy and autotrophy, but further research is needed to understand the mechanism(s) underlying CO_2 assimilation and its relationship with CH_4 oxidation to provide guidance for metabolic engineering efforts in these autotrophic methanotrophs.

8.5 Conclusions

Resurgent interest in CH_4 capture and conversion, coupled with advances in genomic and genetic tool development, will undoubtedly usher in a new era for methanotrophic metabolic engineering. In this chapter, we have highlighted historic and recent progress in this space and presented a series of metabolic engineering tools and strategies aimed at overcoming techno-economic hurdles associated with methanotrophic biocatalysis for targeted production of fuel intermediates and high-value coproducts, including bypass of pyruvate decarboxylation, targeted removal of competitive carbon pathways, and incorporation of CO_2-utilization machinery.

We note that this chapter represents neither an exhaustive summary of metabolic engineering successes to date nor a comprehensive evaluation of potential future strategies. Indeed, gas fermentation engineering and reactor optimization, development of systems biology and bioinformatic tools, and an essential fundamental elucidation of mechanisms governing methanotrophic physiology are beyond the scope of this chapter, but will all play a critical role in bringing to bear the full potential of industrial methanotroph deployment and will iteratively inform metabolic engineering strategies. Additionally, we have primarily focused upon generation of fuel and chemical intermediates for industrial manufacturing pursuits, using aerobic methanotrophic bacteria; methanotrophs hold promise for an array of additional applications, including pharmaceutical, nutraceutical, and agricultural deployment, and anaerobic systems also offer potential candidates for deployment, which will necessitate unique metabolic engineering strategies and considerations. Regardless of application, the potential for methanotrophic biocatalysis is clear, and emerging metabolic engineering capabilities and strategies in these organisms will play a key role in their development.

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Synthetic Methylotrophy: Past, Present, and Future

9

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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).

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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_9

9.1.2 C1 Compounds Utilized by Methylotrophs

The C1 compounds used by natural methylotrophs include methane and methanol, and also methylamines (di-, tri-, and tetramethylamine), formate, formamide, chloromethane, and dichloromethane. While bacterial methylotrophs typically are capable of growing on a variety of C1 compounds—with the exception of methanotrophs, which are mostly dedicated to methane conversion—eukaryotic methylotrophs 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 methylotrophic 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) methylotrophs. 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 α, β, and γ (Merkx 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.,

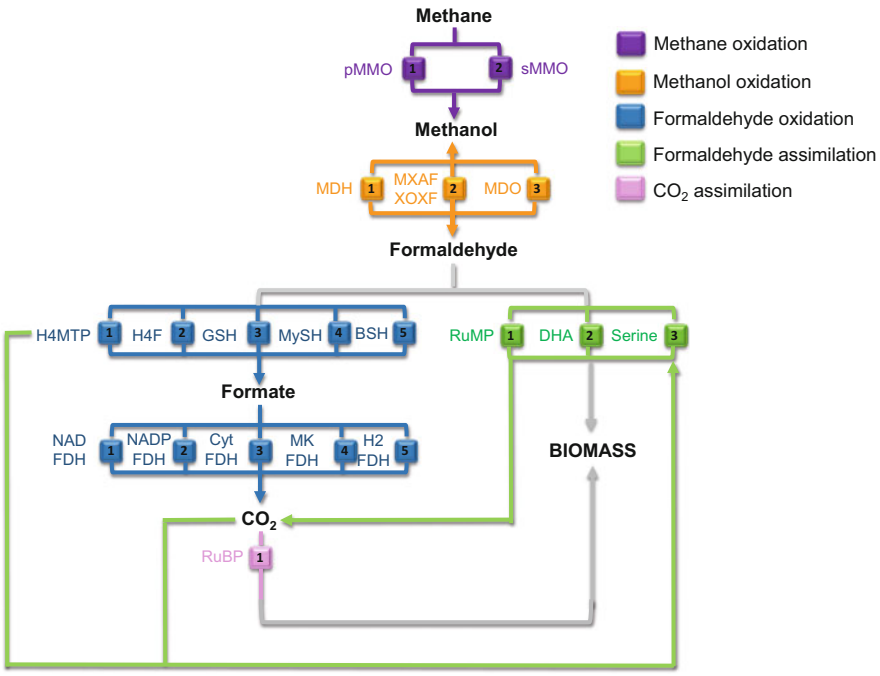

Fig. 9.1 A simplified diagram showing the major metabolic modules involved in methylotrophy. See section "Natural Methylotrophy" for more details

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^{2+} (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_2 (dissimilation), sometimes even within one organism (Vorholt 2002). They comprise linear cofactor-dependent pathways, such as the tetrahydromethanopterin (H_4MPT) or tetrahydrofolate (H_4F)-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_2 via formate dehydrogenases (FDHs). A large number of FDHs exist that differ by the cofactor they use (i.e., NAD, NADP, cytochrome, menaquinone, H_2) (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_2 (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_2. 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 bisphosphate (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_4F catalyzed by serine transhydroxymethylase yielding serine from glycine. In addition, CO_2 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^+ variant) (Chistoserdova 2011) or by the ethylmalonyl-CoA pathway (EMCP) in serine cycle methylotrophs that lack ICL (i.e., ICL^- 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_2, 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 bisphosphate 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_2 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 Methylotrophy

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^3 (Haynes and Gonzalez 2014). This natural gas consists primarily of methane (CH_4), and the most important application of natural gas is the conversion to synthesis gas (i.e., syngas), a mixture of CO and H_2 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_2) 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_2 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 γ-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 Methylotrophs

Methylotrophy 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-methylotrophic hosts.

A number of biochemical and practical considerations have to be taken into account for the design of synthetic methylotrophic 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 methylotrophy metabolism and emphasize the various levels of biochemical and metabolic complexity to engineer synthetic methylotrophs. 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-methylotrophic 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 Methylotrophs

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 methylotrophy 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}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}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 methylotrophic 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
E. coli	*smmo* operon (mca)	×	×	×	West et al. (1992)
Methanotrophs containing only pMMO	*smmo* (mca and mtri)	×	×	×	Lloyd et al. (1999), Murrell (2002), Wood (2002)
Rhodococcus erythropolis	pmmo gene cluster (mca)	×	×	×	Gou et al. (2006)
E. coli	*pmmo* gene cluster (mtri)	×	×	×	Balasubramanian et al. (2010)
Cell-free system	×	*mdh* (bmet)	*hps* (mca)	*phi* (mfa)	Burgard et al. (2014)
E. coli deleted for native formaldehyde oxidation pathway	×	*mdh2* (bmet)	*hps* (bmet)	*phi* (bmet)	Brautaset et al. (2013)
	×	*mdh* (bste)	*hps* (mgas)	*phi* (mgas)	Papoutsakis et al. (2015)
	×	*mdh* (bste)	*hps* (bmet)	*phi* (bmet)	Whitaker et al. (2017)
	×	*mdh3* (bmet)	*hps* (mgas)	*phi* (mgas)	Price et al. (2016)
E. coli	×	*mdh2* (bmet)	*hps* (bmet)	*phi* (bmet)	Muller et al. (2015b)
C. glutamicum deleted for native formaldehyde oxidation pathway	×	*mdh2* (bmet)	*hps* (bmet)	*phi* (bmet)	Brautaset et al. (2013)
	×	*mdh* (bmet)	*hxlA* (bsu)	*hxlB* (bsu)	Lessmeier et al. (2015), Witthoff et al. (2015)
B. subtilis overexpressing genes encoding for Ru5P regeneration enzymes	×	*mdh3* (bmet)	*hxlA* (bsu)	*hxlB* (bsu)	Brautaset et al. (2013)
P. putida	×	×	*hps* (bbri)	*phi* (bbri)	Koopman et al. (2009)
S. cerevisiae	×	*mdh* (bmet)	*hxlA* (bsu)	*hxlB* (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 gastri,* 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 methylotrophic *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}C-labeling of a specialty chemical from ^{13}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 methylotrophs 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}C-label from ^{13}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}C-labeling of cadaverine from ^{13}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}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}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. stearothermophilus* (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 methylotrophs 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_2 through the host's endogenous metabolic routes to avoid accumulation. In methylotrophic *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 methylotrophic *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 methylotrophs 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 ed 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_2 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.

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Engineering Soluble Methane Monooxygenase for Biocatalysis

10

Thomas J. Smith and Tim Nichol

10.1 Introduction

Oxidation of methane to methanol is the critical defining step of aerobic methanotrophic metabolism. It achieves activation of methane, the most unreactive of aliphatic hydrocarbons, and yields methanol as the sole oxidation product. Two separate enzyme systems are able to catalyse this reaction (Smith and Murrell 2009; Hakemian and Rosenzweig 2007), soluble methane monooxygenase (sMMO) and particulate methane monooxygenase (pMMO). Both enzymes are of substantial interest as biological catalysts, and there has been considerable progress in terms of understanding the catalytic properties of both enzymes and the applications that both may have in biocatalysis. They are also the gateway to carbon entering the carbon assimilation pathways of methanotrophs and hence are key to biological conversion of methane to biomass (for fish, animal, or human food materials) and other metabolites such as polyhydroxyalkanoates, engineered metabolites and recombinant proteins (Jiang et al. 2010). To date sMMO has received more attention than pMMO, in part because it has been easier to study (as a soluble rather than membrane-associated enzyme) and also because it has a substantially broader substrate range than pMMO and hence is more attractive as a versatile oxygenation catalyst.

In addition to its potential usefulness in converting methane to methanol biotechnologically (a reaction that cannot currently be achieved in a single step under ambient conditions via chemical catalysis), sMMO is known to oxidise a wide diversity of hydrophobic molecules. It has more than 100 documented substrates, including many hydrocarbons and chlorinated hydrocarbons (Lipscomb 1994; Smith and Dalton 2004) that range in size from methane to polychlorinated biphenyls (Table 10.1), making sMMO arguably the most versatile oxygenation biocatalyst.

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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_10

Table 10.1 Selected substrates of wild-type sMMO and the products formed

Substrate	Major products detected (mole percentages in parentheses)	Specific activity (nmol of product min^{-1} mg^{-1})	Type of assay*
Alkanes			
Methane	Methanol	84	SE[a]
Ethane	Ethanol	68	SE[a]
Propane	Propan-1-ol (39); propan-2-ol (61)	69	SE[a]
Butane	Butan-1-ol (54); butan-2-ol (46)	77	SE[a]
Pentane	Pentan-1-ol (28); pentan-2-ol (72)	73	SE[a]
Octane	Octan-1-ol (9); octan-2-ol (91)	9	SE[a]
2-methylpropane	2-Methylpropan-1-ol (70); 2-methylpropan-2-ol (30)	33	PP[b]
Alkenes			
Ethene	Epoxyethane	148	SE[a]
Propene	1,2-Epoxypropane	83	SE[a]
But-1-ene	1,2-Epoxybutane	33	PP[b]
Monoaromatics			
Benzene	Phenol	74	SE[c]
Toluene	Benzyl alcohol; *p*-cresol	53	SE[a]
Ethylbenzene	1-Phenylethanol (30); 4-hydroxyethylbenzyene (70)	18.7	SE[c]
Styrene	Styrene oxide	82	SE[c]
Diaromatics			
Naphthalene	1-Naphthol; 2-Naphthol		W[d]
Biphenyl	2-Hydroxybiphenyl (9); 3-Hydroxybiphenyl (1); 4-Hydroxybiphenyl (90)		W[e]
2-Chlorobiphenyl	Hydroxychlorobiphenlys		W[e]

*SE, soluble extract; PP, pure protein; W, whole cells
[a]Colby et al. (1977)
[b]Green and Dalton (1989)
[c]Burrows et al. (1984)
[d]Brusseau et al. (1990)
[e]Lindner et al. (2000)

The work towards producing mutants of sMMO has been driven to a large extent by interest in modifying the properties of the enzyme for specific biotransformation and also to understand at the molecular level the unique properties of the enzyme, which is able to accommodate so wide a range of substrates in its active site and to perform such chemically challenging oxidation reactions.

New catalysts for regiospecific oxygenation of unfunctionalised C–H bonds are in demand because there is a lack of workable catalytic technology of any kind, chemical or biological, to perform such transformations (Lewis et al. 2011). One particular challenge in harnessing the wide substrate range of sMMO is therefore to

increase its regioselectivity since, where a number of carbon atoms are available in a substrate, the wild-type sMMO usually yields a mixture of products (Table 10.1). Similarly, the wild-type enzyme generally has low enantioselectivity in producing products containing chiral centres (such as 1,2-epoxypropane from propene).

A whole-cell biocatalyst containing sMMO has been tested on the pilot industrial scale for production of low molecular weight racemic epoxides from the corresponding alkenes, using methanotroph cells in a two-stage reactor system to avoid problems with the toxicity of the epoxide product (Richards et al. 1994). This was tested on a pilot scale in the 1990s and gave yields of racemic epoxides up to 250 g L^{-1} day^{-1} at a cost comparable to the existing chemical manufacturing process. Hence, a system using a recombinant sMMO to produce a product of sufficiently high value could be a commercial success. Results from mutagenesis are also needed to complement extensive structural and spectroscopic characterisation that has been carried out on the wild-type enzyme and its reaction intermediates.

This chapter describes the expression systems for the active site containing hydroxylase component of sMMO, with particular detail on the homologous expression system that has been used to construct and characterise a number of mutants of the enzyme and the use of this system towards production of commercially useful biocatalysts.

10.2 Structure, Biochemistry and Regulation of sMMO

sMMO is a multicomponent oxygenase system with a nonheme dinuclear iron-active centre (DeWitt et al. 1991) that belongs to the soluble diiron monooxygenase (SDIMO) family of enzymes (Coleman et al. 2006) also termed the bacterial multicomponent monooxygenases (BMMs) (Leahy et al. 2003). sMMO comprises three components (Fig. 10.1) and is encoded by the six-gene operon *mmoXYBZDC*. The 250-kDa hydroxylase (encoded by genes *mmoX*, *Y* and *Z*) has an $(\alpha\beta\gamma)_2$ structure, in which each α (MmoX) subunit contains a μ-hydr(oxo)-bridged

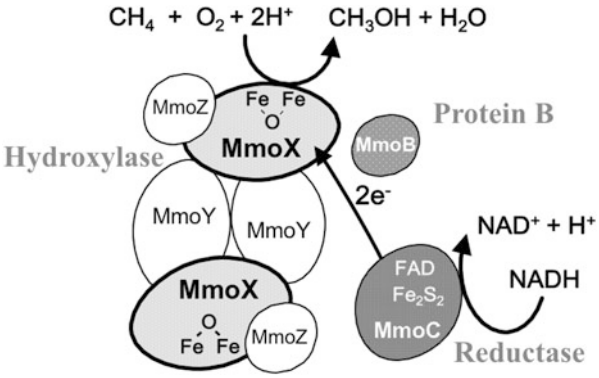


Fig. 10.1 The sMMO enzyme complex

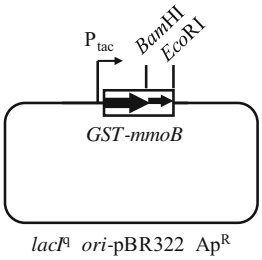
binuclear iron centre that is the site of oxygen and substrate activation. The 37-kDa reductase (encoded by *mmoC*) has FAD and Fe_2S_2 prosthetic groups and supplies reducing equivalents from NAD(P)H to the hydroxylase. Protein B, or the coupling/gating protein (encoded by *mmoB*), is a 15-kDa polypeptide that has no prosthetic groups, binds to the hydroxylase and is necessary for efficient sMMO catalysis (Fox et al. 1991; Lipscomb 1994; Smith and Dalton 2004). MmoD, also known as OrfY, is not part of the mature enzyme complex but may be involved in assembly of sMMO (Merkx and Lippard 2002) and regulation of MMO expression (Semrau et al. 2013).

sMMO is found in a number of obligate methanotrophs that can express either form of MMO depending on the copper-to-biomass ratio of the culture, among which the type II methanotroph *Methylosinus trichosporium* OB3b and the type X methanotroph *Methylococcus capsulatus* (Bath) are the best characterised. sMMO is also found in the facultative methanotrophs of the genus *Methylocella*, which possess sMMO but not pMMO (Dedysh et al. 2005). In the obligate methanotrophs that can express either form of the enzyme, the copper-dependent pMMO is expressed at high copper-to-biomass ratio and sMMO (which does not contain copper) is expressed at low copper-to-biomass ratio (Stanley et al. 1983). Regulation of sMMO and pMMO expression by copper ions is a complex process that that has not yet been completely defined at the molecular level, though it has recently been shown that the copper-sequestering molecule methanobactin and MmoD, which has DNA- and copper-binding properties, are involved in regulating the switch (Semrau et al. 2013). The natural regulation of sMMO by copper ions has been found to be a workable system for expressing recombinant enzymes in the homologous expression system, as detailed below.

Fast reaction kinetics have been used to characterise the intermediates in the sMMO catalytic cycle. Of particular interest is the intermediate compound Q, which is the kinetically competent form of the diiron site that reacts with methane to produce methanol and has an Fe^{IV} 'diamond core' structure (Shu et al. 1997; Banerjee et al. 2015). The manner in which particular components of the protein scaffold interact to produce and protect intermediate Q remains a largely open question.

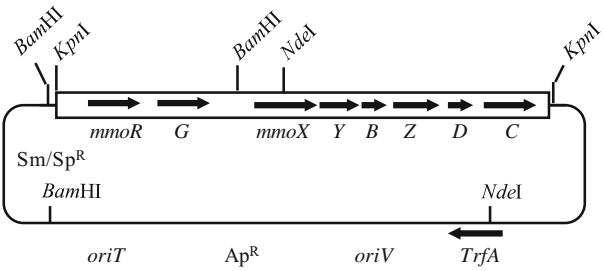
10.3 Heterologous Expression Systems

The protein B and reductase components of sMMO are each active when expressed as single-gene constructs in *E. coli*. Native protein B, both in vivo and in vitro, is subject to proteolysis (which is at least in part an autocatalytic reaction) near the N-terminus terminus, which results in the inactive truncated protein B' that may constitute a regulatory mechanism for sMMO at the enzyme level (Callaghan et al. 2002; Brazeau et al. 2003). When expressed in *E. coli*, the protein can be expressed as a fusion to an affinity tag. If glutathione S-transferase (GST) is attached at the N-terminus (as in plasmid pGEX-MmoB shown in Fig. 10.2), the fusion protein is fully active and is protected against proteolytic inactivation (Lloyd et al. 1997).

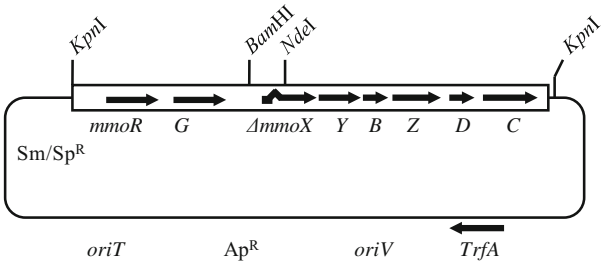


pGEX-MmoB

pGEX-MmoB (vector pGEX-2T), producing in-frame translational fusions of the protein B component, in *E. coli* under IPTG control. The vector provides a thrombin cleavage site between the fused proteins, although the GST-protein B fusion is fully active and used with the tag attached.



pTJS175 for transfer by conjugation and expression of sMMO in *Ms. trichosporium* sMMO-minus strain mutant F or SMDM



pT2ML for transfer by conjugation and expression of sMMO in *Ms. trichosporium* sMMO-minus strain mutant F or SMDM and exchange of mutant and wild-type *mmoX* genes in a single cloning step using *Bam*HI and *Eco*RI. This version of the plasmid has a partially deleted version of *mmoX* that allows recombinants containing the full-size gene to be easily identified after cloning.

Fig. 10.2 A selection of plasmids used for expression of sMMO (Not drawn to scale)

Other systems for expressing protein B in *E. coli* have used a C-terminal 6His tag (Callaghan et al. 2002) or no tag (Chang et al. 1999; Coufal et al. 2000). *E. coli* expression systems have permitted mutagenesis to explore the role protein B in determining hydroxylase oxidation state and access to the hydroxylase active site (Wang and Lippard 2014) and the role of sequence around the N-terminus in determining truncation and the role of truncation in activity (Lloyd et al. 1997; Callaghan et al. 2002; Brazeau and Lipscomb 2003).

The sMMO reductase is active when expressed in *E. coli* (West et al. 1992), and an *E. coli* system has also been used to express the FAD-binding domain of the reductase (Chatwood et al. 2004).

Whilst the diiron-containing oxygenase components of a number of other SDIMOs, particularly those with aromatic compounds as their natural substrates (e.g. toluene 4-monooxygenase [Bailey et al. 2008]), have been successfully expressed in *E. coli*, the oxygenase components of SDIMOs of classes 3 (including sMMO) and 4 (including the alkene monooxygenase of *Rhodococcus rhodochrous* B-276) (Coleman et al. 2006) have not to date been obtained in active form in *E. coli*, possibly due to problems with correct folding of the enzyme. In the chromosome of *Ms. trichosporium* OB3b, the sMMO operon is adjacent to the divergently transcribed cluster of two genes, a putative GroEL-like chaperone gene (*mmoG*) and a putative sigma-54-dependent transcriptional regulator (*mmoR*), and inactivation of either gene by marker exchange mutagenesis abolishes both transcription and activity of sMMO (Stafford et al. 2003).

Wood and colleagues have obtained active expression of sMMO from *Ms. trichosporium* OB3b by using alternative expression hosts. sMMO activity, as judged by detectable degradation of trichloroethene (TCE, a substrate for sMMO), was detected from clones of the sMMO genes in *Pseudomonas putida*, *Agrobacterium tumefaciens* and *Rhizobium meliloti* (Jahng and Wood 1994; Jahng et al. 1996). Expression in *P. putida* gave an activity of 5 nmol min^{-1} (mg of protein)$^{-1}$ with trichloroethene as the substrate (Jahng and Wood 1994).

10.4 Methanotroph Expression Systems

The hypothesis underlying the use of a methanotroph expression system is that, whatever factors are necessary for expression of the sMMO genes and assembly of the polypeptides and iron cofactors into a functional enzyme, these will be present in a host that can naturally produce the enzyme in an active form. One approach is to express a recombinant copy of the sMMO operon in a methanotroph that naturally produces only pMMO, presuming that such methanotrophs also possess the factors needed to express sMMO. When the sMMO operon of *Ms. trichosporium* OB3b was cloned into the pMMO-only methanotrophs *Methylomicrobium album* BG8 and *Methylocystis parvus* OBBP, detectable sMMO activity was found, though engineering expression of sMMO (which was induced by low copper levels even in this system) was challenging since the strains retained a substantial requirement of copper for growth (Lloyd et al. 1999a).

The sMMO expression system that has been most productive to date is based upon homologous expression in strains of *Ms. trichosporium* in which the chromosomal copy of the sMMO operon has been inactivated by marker exchange mutagenesis (Lloyd et al. 1999b; Smith et al. 2002). Recombinant (wild-type or mutant) sMMO genes can then be introduced on plasmids via conjugation from a suitable *E. coli* donor strain. The first such system used a mutant of *Ms. trichosporium* OB3b termed mutant F (Martin and Murrell 1995), in which a 1.2-kb fragment internal to *mmoX* (encoding the active site containing α-subunit of the hydroxylase) is replaced by a kanamycin-resistance cassette. In order to obtain expression of a recombinant sMMO, a clone containing *mmoX* and its natural promoter alone was not sufficient to restore sMMO expression (Smith et al. 2002), and so plasmid clones containing the whole sMMO-encoding operon plus *mmoR* and *mmoG* have been used in all subsequent studies. In this system sMMO remains under the control of its natural promoter and so is induced by low copper-to-biomass ratio of the culture, and so the enzyme can be induced either in a fermentor by growth to a sufficiently high density or in flask or plate cultures with medium containing a sufficiently low concentration of copper (Smith and Murrell 2011). The enzyme was expressed at levels comparable to the wild-type strain, and the wild-type hydroxylase purified from the recombinant wild type was essentially indistinguishable in terms of its specific activity from the hydroxylase purified from the parental strain *Ms. trichosporium* OB3b, with a specific activity of 244 nmol min^{-1} (mg of protein)$^{-1}$ using the standard assay substrate propene (Smith et al. 2002). In all cases, growth begins using copper-dependent pMMO and then switches to sMMO as the copper-to-biomass ratio decreases with growth of the culture. This allows expression even of mutants of sMMO that are inactive.

Use of mutant F as the expression host is limited to mutants of the hydroxylase where the mutations lie in the part of *mmoX* that is deleted in the chromosome (corresponding to amino acids Val 112-Thr 508 in the α-subunit), since recombinant genes with mutations outside this region could in principle be repaired by recombination with the remnant of the chromosomal copy of the sMMO operon. The more recently constructed strain *Ms. trichosporium* SMDM (Borodina et al. 2007) is deleted in the whole of the sMMO-endoding operon except for the 5' region of *mmoC* (encoding the C-terminal region of the reductase) and so can be used for expression of mutants anywhere in the structure of the hydroxylase. In the absence of complementing recombinant sMMO genes, SMDM has a perturbed copper switch in which the expression of the pMMO genes is greatest when no copper is added to the medium (Semrau et al. 2013), indicating that a component of the sMMO operon is needed for functioning of the copper switch. In contrast, sMMO (wild-type or mutant) in a plasmid-complemented strain SMDM (Borodina et al. 2007) is induced by low copper-to-biomass ratio in a similar way to the wild-type and mutant F.

The plasmid system for homologous expression has been similarly developed to make it easier to use. All plasmids that have been used for expression of mutant hydroxylases have been based upon pJB3Km1 (Blatny et al. 1997), which has a broad host-range plasmid replicon and origin of conjugative transfer. The initial plasmid used for expression of sMMO mutants (pTJS175 in Fig. 10.2) required

multiple subcloning steps for each mutant (Smith et al. 2002). Successive changes have been made to the plasmid, resulting in plasmid pT2ML (Fig. 10.2), which allows cloning of PCR-derived fragments of *mmoX* in a single-cloning step and easy identification of recombinants via a change in the size of a PCR product spanning the *mmoX* gene (Lock et al. 2017).

10.5 Results from Mutagenesis to Date

Mutants of the hydroxylase component published to date are listed in Table 10.2.

Table 10.2 Summary of altered properties of the mutants

Residue (hydroxylase α-subunit)	Structural context	Mutation	Effect	Conclusion
Arg 98[a]	Periphery of enzyme; salt bridge to Asp α-365	Leu	Increased activity and regioselectivity with biphenyl	Role in determining precision of holding large substrate in active site
Leu 110[b]	Active site pocket hydrophobic; proposed gating role	Gly	Relaxation of regioselectivity of aromatic substrates	Mutations diminish precision of holding aromatic substrates in active site
		Cys		
		Arg		
		Tyr		
Cys 151[c]	Protonated residue in otherwise hydrophobic active site cavity. Equivalent to Tyr 122 in ribonucleotide reductase	Tyr	Very unstable; possibly inactive	Structurally important; no essential role for radical at this position
		Glu	Unstable; detectable activity with naphthalene	
Phe 192[a]	Active site pocket; hydrophobic	Ile	Increase in side chain hydroxylation of toluene; diminished regioselectivity with biphenyl substrate	Involvement in regioselectivity
Thr 213[c]	Protonated residue in otherwise hydrophobic active site cavity. Analagous position to Thr 252 in P450.	Ala	Trace of activity with naphthalene	Hydroxyl group appears preferable at this position
		Ser	Active and stable; greater reduction in activity towards propene than toluene	
Ile 217[a]	Active site pocket; hydrophobic	Ala	Diminished regioselectivity with biphenyl substrate	Involvement in regioselectivity

[a]Lock et al. (2017)
[b]Borodina et al. (2007)
[c]Smith et al. (2002)

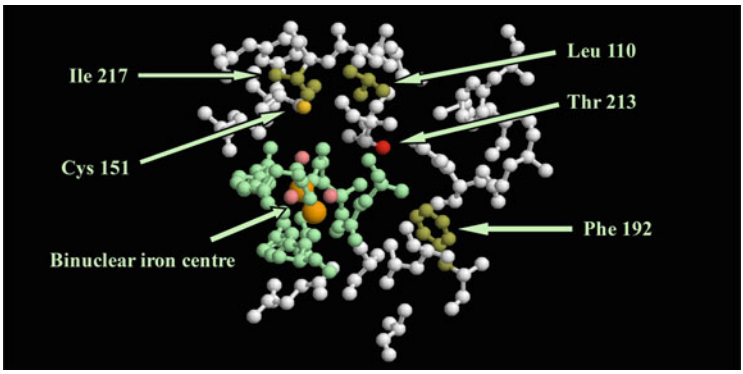


Fig. 10.3 Sites in and around the sMMO hydroxylase active site mutated to date. Numbered labelled residues are the active site residues mutated to date. The iron atoms of the diiron site are shown as large orange spheres, diiron centre-associated water molecules as pink spheres and the ligands of the diiron site in light green. The remaining residues shown constitute the substrate-binding pocket, within which the S^{γ} of Cys151 and the O^{γ} of Thr 213 (which are the only protonated residues within the active site) are shown as yellow and red spheres, respectively. Hydrophobic residues that have been mutated are shown in olive green

10.5.1 Mutants of Cys 151 and Thr 231

The initial sites chosen for mutagenesis were selected in order to test the roles of two hydrophilic residues in the active site of the hydroxylase. The active site pocket within the hydroxylase is highly hydrophobic apart from the residues that ligate the diiron centre and two residues with protonated heteroatom side chains, Cys 151 and Thr 213 (Fig. 10.3).

In alignments of SDIMOs and other homologous diiron proteins, the amino acid at the position equivalent to Cys 151 in sMMO correlates with the function of the enzymes. All known sMMOs have cysteine here, monooxygenases that naturally epoxygenate alkenes have glutamate or aspartate (i.e. a carboxyl side chain) (Saeki and Furuhashi 1994; Zhou et al. 1999), monooxygenases that perform ring hydroxylations of aromatic compounds have glutamine (Johnson and Olsen 1995), and ribonucleotide reductases have tyrosine (Sjöberg 1997). Also, in the R2 subunit of class I ribonucleotide reductase, which is also homologous to sMMO (Nordlund et al. 1992), an O_2-dependent one-electron oxidation of Tyr 122 (equivalent to Cys 151 in sMMO) produces a stable tyrosyl radical that initiates the radical-dependent reduction of ribonucleotides at the active site of the R1 subunit of this enzyme (Sjöberg 1997).

The C151E mutant showed slight activity towards the diaromatic substrate naphthalene, strongly suggesting that C151 was not involved in radical chemistry. No activity could be detected for the C151Y mutant and neither mutant strain produced sufficient hydroxylase for it to be detected via SDS-PAGE after growth of the culture in a fermentor on methane. This suggested that both mutant hydroxylases were either synthesised at low levels or were unstable. Intriguingly,

the phenotypes of the strains expressing the mutant sMMOs support the correlation between the function of binuclear iron-active site and the amino acid at the position 151 and its equivalents. Mutation of Cys 151 to Glu, as found in alkene monooxygenase of *R. rhodochrous* B-276 (Saeki and Furuhashi 1994), preserved oxygenase activity, whereas mutation of Cys 151 to Tyr, as in ribonucleotide reductase (Sjöberg 1997) (which is not a monooxygenase), abolished it. In a study using toluene 4-monooxygenase, the equivalent residue Gln 141 was mutated to cysteine (as in sMMO), and this also preserved monooxygenase activity (Pikus et al. 1997).

The activity of T213A and T213S towards naphthalene showed that this residue was also not essential for catalytic activity of sMMO. The instability of soluble cell extract preparations of the T213A mutant and the stability and activity of T213S mutant led to the suggestion of the importance of an OH group at this position for stability of the enzyme. However, the activity of the T213S mutant within soluble cell extract preparation enabled the first purification of a stable site directed mutant of sMMO (Smith et al. 2002).

10.5.2 Mutants at Leu 110

The hydrophobic substrate-binding site of sMMO is adjacent to the binuclear iron-active centre and is deeply buried in the 250-kDa $(\alpha\beta\gamma)_2$ hydroxylase component (Elango et al. 1997; George et al. 1996; Rosenzweig et al. 1993), presumably to prevent the solvent from quenching the highly oxidising di-ferryl (Fe^{VI}_2) intermediate Q (Shu et al. 1997) that is needed to oxygenate methane and other recalcitrant substrates. Access to the active site is likely to be via another hydrophobic pocket, cavity two, which is part of a chain of buried cavities that communicate between the active site and the solvent. Between cavity two and the active site lies the 'leucine gate', residue Leu 110 (Fig. 10.4). Different crystal forms of the hydroxylase differ in the conformation of Leu 110, such that in the crystal state it can either block the pathway between cavity two and the active site or (in the alternative conformation) open a 2.6-Å diameter channel between the two cavities. A larger conformational change, such as may be caused by interaction with the other components of the sMMO complex, could open this 'leucine gate' further, to allow passage of substrates and products (Rosenzweig et al. 1997), reasonably acting to control access of substrates to the active site.

The homologous expression system described above was used to carry out site directed mutagenesis on the Leu110 residue of the sMMO hydroxylase α-subunit. Site-directed mutagenesis was used to create mutants L110R and L110Y with larger side chains and L110C and L110G with smaller side chains at this position. These mutants were tested for activity against a number of monoaromatic and diaromatic substrates. Activity against the monoaromatic substrates toluene and ethyl benzene and the diaromatic biphenyl showed a relaxed regiospecificity for all mutants including a number of oxidation products at the 2- and 3-position of the benzene ring not seen with the wild-type enzyme. Mutation to residues Arg and Tyr resulted

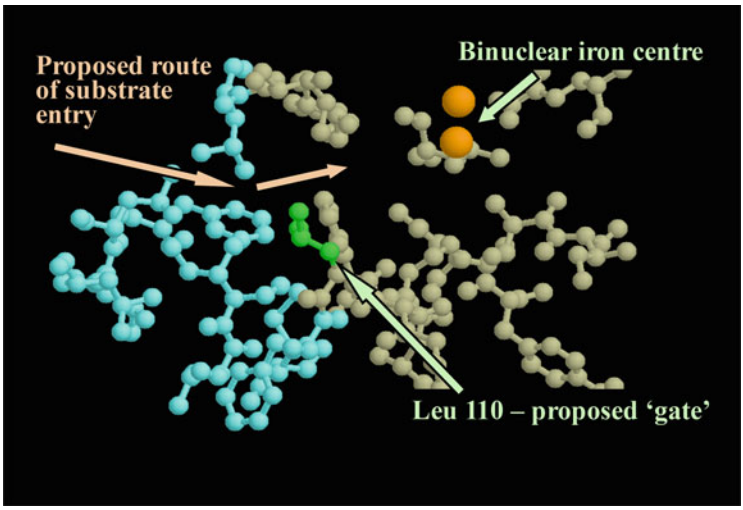


Fig. 10.4 Position of Leu 110 as a putative gating residue. It was proposed that substrates enter the deeply buried active site pocket (brown) via cavity two (light blue) as indicated by the pink arrows. A conformational change affecting the side chain of Leu 110 (green) was proposed to act as a gate controlling access to and from the active site

in a change in regioselectivity from primarily the 2- to primarily the 1-position with the diaromatic substrate naphthalene. This led to the conclusion that residue Leu110 had a role in determining regioselectivity and that the results of analysing these mutants did not give evidence for a role in controlling the size of substrate that can enter the active site (Borodina et al. 2007).

10.5.3 Mutants to Increase the Regioselectivity of sMMO

In a more recent set of mutants, Phe 192 (Fig. 10.3) was chosen as a site for mutagenesis because it is positioned at the same end of the substrate-binding pocket as the diiron centre and, together with another phenylalanyl residue (Phe 188), interacts with an acetate ion in the structure of sMMO from *Methylococcus capsulatus* (Bath) (Rosenzweig et al. 1995). Both phenylalanyl residues are conserved in the sMMO from *M. trichosporium* OB3b that was used in the mutagenesis experiments and may be critical in determining the hydrophobic landscape next to the diiron centre where substrates bind. The mutation F192I was designed based on sequence alignments, where this position is occupied by Ile 179 in the homologous toluene 4-monooxygenase (T4MO) (Leahy et al. 2003) that naturally oxygenates monoaromatic hydrocarbons. Another residue investigated was Ile 217, which is within the substrate-binding pocket (Fig. 10.3), though distal to the diiron centre and was converted to the less bulky alanyl residue, with the initial intention of producing a mutant that might (unlike the wild type) be able to

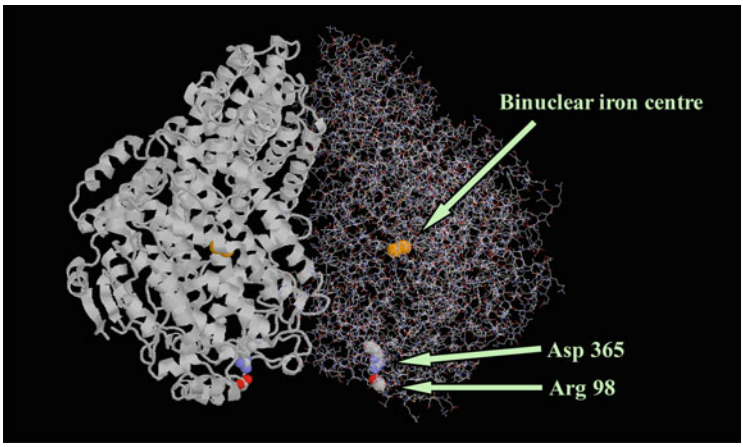


Fig. 10.5 Position of mutation site Arg 98. The $(\alpha\beta\gamma)_2$ structure of the hydroxylase showing the positions within the α-subunit of Arg 98 and Asp 365 (with which it forms an ionic interaction). The left hand αβγ protomer of the hydroxylase indicates the positions of the secondary structure elements, and the right hand protomer is shown as a wireframe

accommodate and oxygenate triaromatic hydrocarbons in its active site. Three ionic networks, near to the upper apex of the αβγ protomer of the sMMO hydroxylase, were identified as possible mutagenesis sites that could be used to manipulate the conformational flexibility of the enzyme. It was also reasoned that mutations at these positions might modulate substrate access into the active centre, via the chain of internal cavities in this region within the protein that has been experimentally tracked by xenon gas in crystals of the homologous sMMO of *Methylococcus capsulatus* (Bath) (Whittington et al. 2001). It is also known that this aperture is opened by binding of protein B to the hydroxylase (Lee et al. 2013; Wang and Lippard 2014). In the *M. trichosporium* OB3b enzyme studied here, these networks comprise the R98/D365 ion pair, the D164/E101/K104/R360 network and ion pair between E230 and R12 (of the β-subunit). Here, the role of the R98/D365 ion pair (Fig. 10.5) was tested via the mutation R98L.

With toluene as the substrate, the R98L and I217A mutants gave similar regioselectivities to the wild type, namely a mixture of products with oxygenation on the side chain and at the 4-position, i.e. at the 'ends' of the molecule if the substrate is considered to be a rectangle. The F192I mutant showed a substantial shift in regioselectivity towards the side chain. With the bulkier substrate biphenyl, where hydroxylation by the wild-type sMMO was detectable at the 2- and 4-positions of the molecule, the F192I and I217A mutants showed the appearance of detectable amounts of 3-hydroxybiphenyl not seen with the wild type. The R98L mutant showed a substantial increase in activity (sixfold compared to the wild type, resulting in an activity towards biphenyl of 4.6 nmol min^{-1} [mg of protein]$^{-1}$ in the mutant). The R98L mutant also showed a significant increase in regioselectivity for the 'end' 4-position of biphenyl, giving $97.9 \pm 0.51\%$ pure 4-hydroxybiphenyl as the product,

compared with the wild type where the proportion of 4-hydroxybiphenyl was 93.9 ± 1.5%, which was significant at the 5% level in an ANOVA test (Lock et al. 2017). These results are the first 'proof-of-principle' experiments illustrating the feasibility of developing sMMO-derived catalysts with improved catalytic precision.

10.6 Conclusions and Future Prospects

Substantial challenges in expression of sMMO have been overcome to allow expression of a number of mutant enzymes that have shed light on the roles of individual residues in the functioning of the enzyme and, most recently, to a mutant that provides proof-of-principle for improving the regioselectivity of sMMO for a specific hydroxylation reaction. Further improvements to the system may enable analysis of larger numbers of mutants, including construction of mutant libraries for directed evolution experiments and for production of 'designer' oxygenases for specific valuable biotransformations. Further pure protein studies, such as fast reaction kinetics, may enable elucidation of the roles of specific residues in the catalytic cycle of this remarkable enzyme, including the key highly oxidising species Q. Furthermore, the ability to produce valuable biocatalysts in a methane-oxidising bacterium from inexpensive biological or fossil methane sources may prove a substantial advantage.

Acknowledgements TJS gratefully acknowledges funding for work on expression and mutagenesis of sMMO from the Biotechnology and Biological Sciences Research Council and the Biomolecular Sciences Research Centre at Sheffield Hallam University.

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Methanol Biosynthesis Using Methanotrophs

11

Toshiaki Kamachi and Ichiro Okura

11.1 Introduction

Methane is the primary component of natural gas, which is a promising alternative fuel source because methane is a renewable energy source and also the "shale gas revolution" has led to major increases in reserves of natural gas. Methane can be produced by anaerobic process called methanogenesis using methanogens (Ferry 2006; Austin and Groves 2011). Liquefaction of methane is needed to utilize methane for ease of storage or transport, but liquefaction of natural gas is uneconomic not only because it requires gas to be cooled to very low temperatures but also transportation or storage facilities needs specially designed refrigerated and insulated tanks. Therefore, the conversion of methane to methanol is desired for the usage of natural gas. However, selective oxidation of methane to methanol is extremely difficult chemistry because the C–H bond in methane has one of the highest bond energies (104 kcal mol^{-1}) among organic substrates. Recently, several groups have reported the direct oxidation of methane to methanol using transition metal catalyst and metalloenzymes (Arndtsen et al. 1995; Periana et al. 1998; Que and Tolman 2008; Caballero and Perez 2013; Shul'pin 2013), but the development of efficient catalyst for direct conversion of methane to methanol is still one of the most challenging subjects in the catalytic chemistry.

Methanotrophs can grow on methane as sole carbon source and energy source. Methane monooxygenase (MMO) in the methanotrophs is the initial enzyme for methane metabolism and catalyzes methane hydroxylation to methanol at ambient

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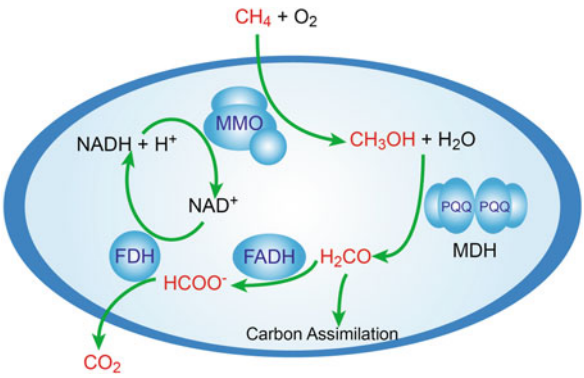
M. G. Kalyuzhnaya, X.-H. Xing (eds.), *Methane Biocatalysis: Paving the Way to Sustainability*, https://doi.org/10.1007/978-3-319-74866-5_11



Scheme 11.1 Methane metabolism in methanotrophs. MMO, methane monooxygenase; MDH, methanol dehydrogenase; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase; PQQ, pyrroloquinoline quinone

temperature and pressure. Therefore, many researchers have studied about MMO's structure, active site, and mechanism of the oxidation of methane to methanol (Balasubramanian et al. 2010; Armstrong et al. 2013; Hakemian and Rosenzweig 2007). In this text, we would like to describe the methanol biosynthesis using *Methylosinus trichosporium* OB3b.

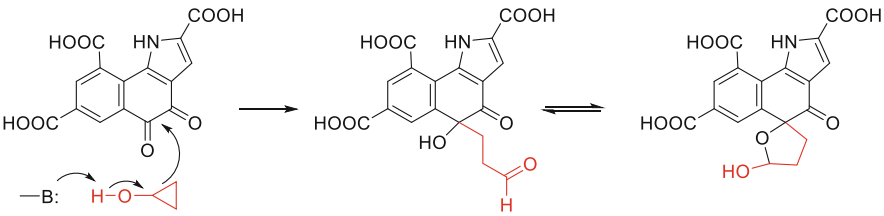
11.2 Biosynthesis of Methanol from Methane

11.2.1 Methanol Synthesis in Batch Reaction

Scheme 11.1 shows the outline of methane metabolism within methanotrophs. Methanotrophs can grow on methane as sole carbon and energy source. Methane is oxidized to methanol by MMO and is subsequently oxidized by methanol dehydrogenase (MDH) to formaldehyde, which is at the diverging point for assimilation and biosynthesis and for catabolism to carbon dioxide for energy source. Oxidation of methane is carried out by MMO according to Eq. (11.1). The oxidation of methane to methanol by MMO requires two electrons and molecular oxygen. NADH is a physiological electron donor for MMO in the microorganisms.

$$CH_4 + O_2 + 2H^+ + 2e^- \underset{MMO}{\rightarrow} CH_3OH + H_2O \qquad (11.1)$$

Purified MMO is not suitable in methanol biosynthesis, because of its instability. Thus, the production of methanol from methane with methanotrophs was tried. Methanol is an intermediate metabolite in methane metabolism, so accumulation of methanol is not achieved by wild-type methanotroph. To prevent further oxidation of methanol, inhibition of MDH is needed. MDH is a quinoprotein that uses 2,7,9-tricarboxypyrroloquinoline quinone (PQQ) as a cofactor (Zheng et al. 2001). PQQ contained in MDH can be irreversibly inhibited by cyclopropanol as shown in Scheme 11.2 (Frank et al. 1989).



Scheme 11.2 Inactivation of PQQ moiety within MDH by cyclopropanol

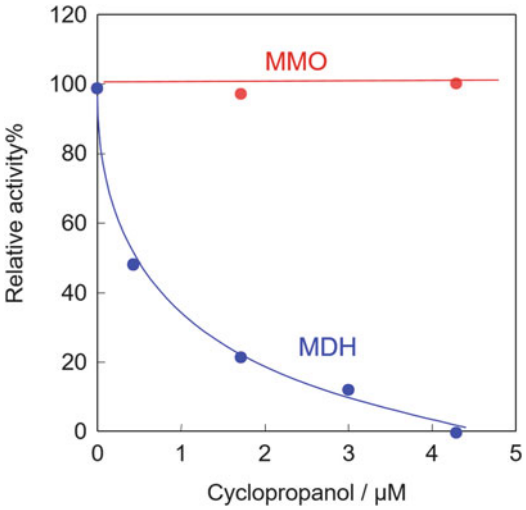


Fig. 11.1 Effect of cyclopropanol on the activity of MMO and MDH

MDH was inhibited by cyclopropanol in a concentration-dependent manner, but MMO activity did not change as shown in Fig. 11.1. So cyclopropanol is a suitable inhibitor for inhibition of MDH for bioconversion of methane to methanol using methanotrophs. To optimize methanol accumulation by *M. trichosporium* OB3b, the effects of cell density, reaction temperature, concentration of sodium formate, concentration of phosphate buffer, pH 7.0, and concentration of cyclopropanol were examined (Takeguchi et al. 1997). The methanol biosynthesis under optimum conditions is shown in Fig. 11.2. By optimizing the reaction condition, the reaction continued for 100 h, and the produced methanol was 152 mmol/g of dry cell. Methanol production yield per consumed methane was 61%. MDH inhibition by cyclopropanol is important for accumulation of methanol, but the depletion of NADH within the cell may occur. Depletion of NADH inhibits the oxidation of methane by MMO because MMO needs electrons from NADH to oxidize methane to methanol. Sodium formate was added as an electron donor in the optimum conditions. Formate can be oxidized to carbon dioxide by formate dehydrogenase (FADH), and regeneration of NADH occurs during reaction. Final concentration of

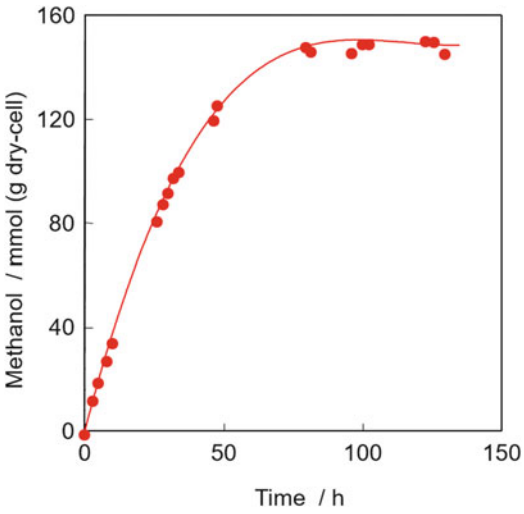


Fig. 11.2 Time course of methanol production by *M. trichosporium* OB3b. Reaction mixture 3.5 mL contains 3.46×10^{-5} g dry cell/mL in 12.9 mM phosphate buffer (pH 7.0) containing 14.3 mM sodium formate and 67.0 nM cyclopropanol. Reaction was carried out at 25 °C

methanol in the reaction mixture was 6 mM. Saturation behavior of the methanol biosynthesis was not due to the deactivation of MMO but the product inhibition of MMO by methanol as mentioned below.

11.2.2 Product Inhibition of Methanol in the Methanol Biosynthesis

Figure 11.3 shows the effect of methanol on the methane oxidation with *M. trichosporium* OB3b. As shown in Fig. 11.3, the rate of methane oxidation was inhibited by increasing methanol concentration in the reaction mixture and was completely inhibited at 10 mM methanol, showing methanol has an inhibitory effect on MMO in *M. trichosporium* OB3b.

11.2.3 Methanol Synthesis in Semicontinuous Reaction

As methanol inhibits the methanol synthesis, a semicontinuous methanol synthesis was tried to remove the produced methanol (Furuto et al. 1999).

In semicontinuous reaction, methanol production by *M. trichosporium* OB3b was carried out as follows. A standard 50 mL capacity ultrafiltration cell was used as a semicontinuous stirred reactor as shown in Fig. 11.4. The sample solution 17.5 mL containing cell suspension treated with cyclopropanol and sodium formate 14.3 mM in 12.9 mM phosphate buffer (pH 7.0) was introduced into the UF cell-attached with ultrafilter Diaflo ultrafilter YM-100(Grace Japan, Amicon). The UF cell was incubated for 5 min at 30 °C, and the reaction was initiated by injecting 12.5 mL of methane into the UF cell with a gastight syringe. After incubation at 30 °C for 90 min, the reaction

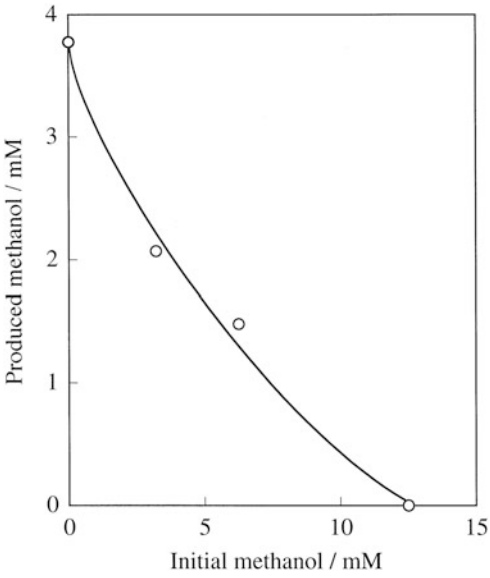


Fig. 11.3 Effect of methanol on methanol synthesis reaction with *M. trichosporium* OB3b. The reaction mixture 3.5 mL contains cell suspension treated with cyclopropanol, methane 112 μmol, oxygen 103 μmol, and sodium formate 14.3 mM in 12.9 mM phosphate buffer pH 7.0

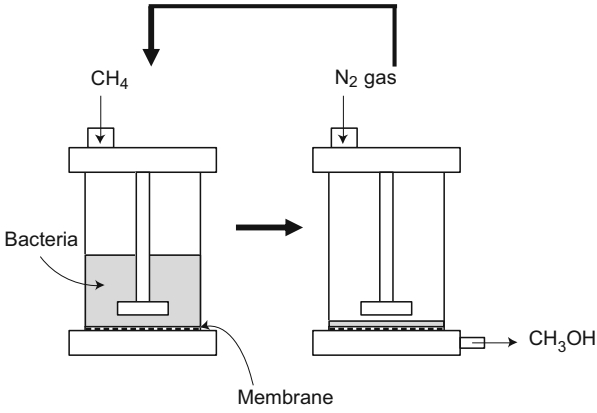


Fig. 11.4 Outline of semicontinuous reactor

mixture was filtrated by nitrogen pressure, leading to separation of produced methanol from cell suspension. The above procedure was repeated several times. Figure 11.5 shows the time dependence of methanol synthesis with *M. trichosporium* OB3b in the semicontinuous reaction. In this reaction, the methanol synthesis was repeated five times for 6 h and had a stationary rate of 3.17 μmol h^{-1} (mg dry cell)$^{-1}$ for 6 h. When the methanol synthesis was repeated five times for 6 h, produced methanol was 36.1 μmol compared to 19.6 μmol in batch reaction under the same conditions.

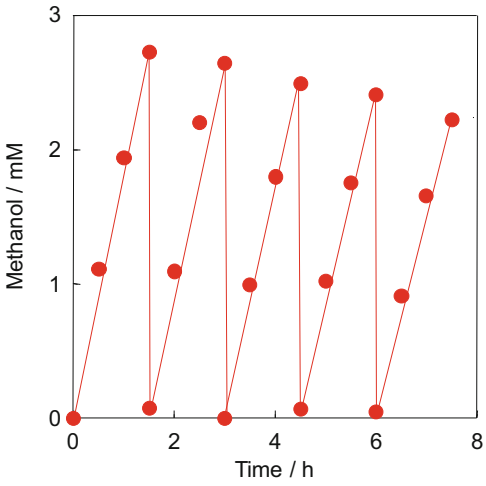


Fig. 11.5 Semicontinuous methanol synthesis with *M. trichosporium* OB3b. The reaction mixture (17.5 mL) contains cell suspension treated with cyclopropanol, methane 560 μmol, oxygen 515 μmol, and sodium formate 14.3 mM in 12.9 mM phosphate buffer pH 7.0

11.3 Characterization of MMO

11.3.1 Purification and Properties of Particulate MMO

There are two well-studied forms of MMO: the soluble form (sMMO) and the particulate form (pMMO). The active site in sMMO contains a di-iron center bridged by an oxygen atom. The active site in pMMO contains copper. Structures of both proteins have been determined by X-ray crystallography; however, the reaction mechanism of pMMO is still poorly understood, partially due to low stability of pMMO after solubilization and purification of pMMO. The purification method of pMMO from *M. trichosporium* OB3b was improved, and purified pMMO retained its activity with duroquinol as a reductant. n-Dodecyl-β,D-maltoside was used for the solubilization of pMMO, and Brij 58 was used for the purification by anion exchange chromatography (Miyaji et al. 2002; Takeguchi et al. 1999).

The purified pMMO monomer (94 kDa) contained only two copper atoms and did not contain iron. The EPR spectrum of the pMMO showed only a typical type II copper signal $g_{\|} = 2.23$, $|A_{\|}| = 18.8$ mT, $g_{\perp} = 2.06$, and nine splitting super-hyperfine structure clearly in the $g_{\perp}$ region as shown in Fig. 11.6. A similar spectrum has been observed for cells or membrane fractions containing pMMO (Zahn and DiSpirito 1996; Yuan et al. 1999; Lemos et al. 2000). *Redox titration experiment of pMMO measured by EPR is shown in Fig. 11.7. Type II EPR signal found* in pMMO was almost eliminated at around 0 mV (vs. NHS), while small EPR signal remained until around −300 mV. From these results, there are at least two types of copper ions in pMMO that differ in the redox potential. These results are consistent with the report by Nguyen et al. that the membrane fraction from *M. capsulatus* Bath containing pMMO showed two copper ion signals imposed at low temperature (Nguyen et al. 1998). Figure 11.8 shows the relationship between $g_{\|}$and $|A_{\|}|$. Red square is the data obtained by previous EPR signal. These parameters are in

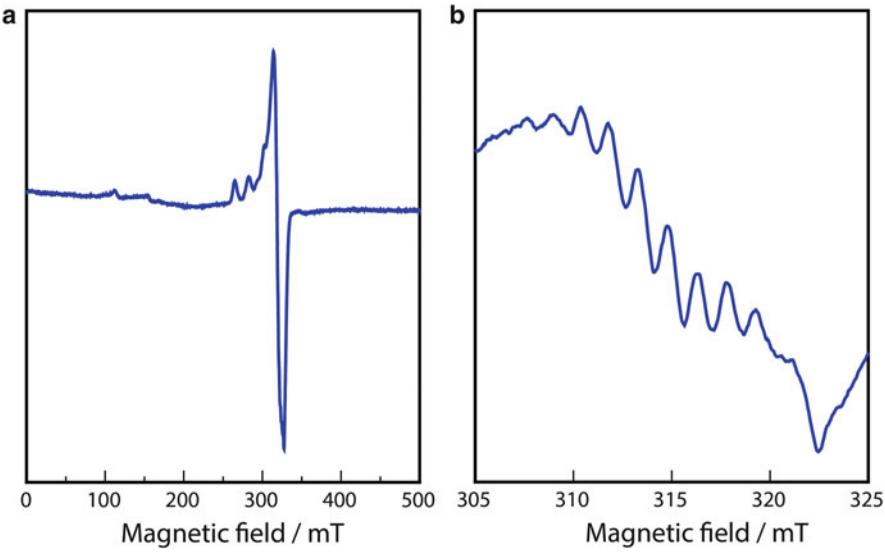


Fig. 11.6 EPR spectrum of pMMO from *M. trichosporium* OB3b. (**a**) Type II copper EPR signal obtained from purified pMMO. (**b**) Enlarged spectrum at $g_{\perp}$region. Nine hyperfine splitting was observed

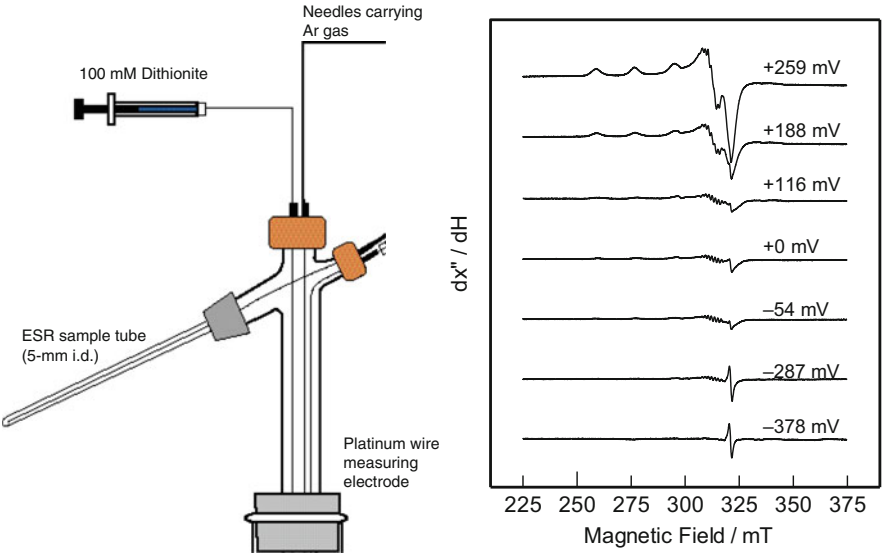


Fig. 11.7 EPR redox titration of pMMO from *M. trichosporium* OB3b. (**a**) Experimental setup for redox titration. (**b**) Dependence of EPR spectra of pMMO on the redox potential

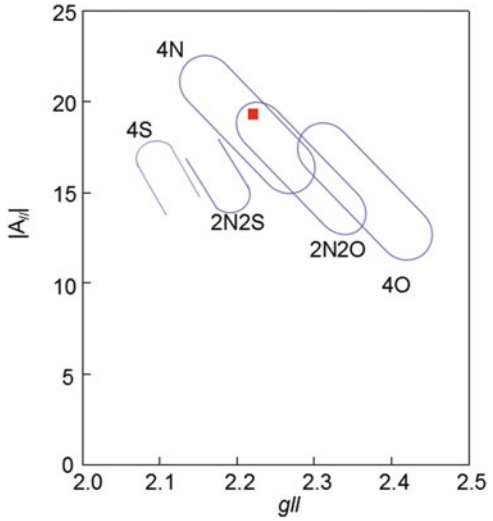


Fig. 11.8 Relationship between $g_{\parallel}$ and $A_{\parallel}$ for type II copper ion found in purified pMMO from M. trichosporium OB3b. Red square is the data obtained by EPR signal shown in Fig. 11.6

agreement with ligations to 4N, 3N1O, or 2N2O. X-ray crystallography data reported by Rosenzweig et al. also support the existence of two types of copper ion site ligated by nitrogen atom-rich surroundings (Balasubramanian et al. 2010; Hakemian et al. 2008). Figure 11.9 shows the structure of pMMO from *M. capsulatus* BATH. According to the crystallographic analyses, the overall architecture of pMMO from the two strains is almost identical. pMMO is a αβγ trimer comprising three subunits (PmoB (α subunit), PmoC (β subunit), and PmoA (γ subunit)). The dinuclear copper site is located in a water-soluble N-terminal subdomain of PmoB, and the mononuclear site is found in a hydrophobic region of pMMO of PmoC. The role of these copper sites remains unclear.

11.3.2 Electron Transfer Chain Within the Cytoplasmic Membrane Containing pMMO

As mentioned above, MMO catalyzes the oxidation of methane to methanol in the presence of an electron donor. NADH is a physiological electron donor for pMMO. When the cytoplasmic membrane is prepared from microorganism, pMMO activity can be measured using NADH as an electron donor. The solubilized or purified pMMO does not catalyze the oxidation of methane using NADH as an electron donor (Miyaji et al. 2002). So electron donor should be changed to duroquinol instead of NADH. Duroquinol is a reduced form of quinone. This indicates the existence of an electron transfer chain from NADH to pMMO. To clarify the existence of the electron transfer chain within the cytoplasmic membrane, inhibition of NADH-dependent MMO activity was carried out. In mitochondria, electrons are

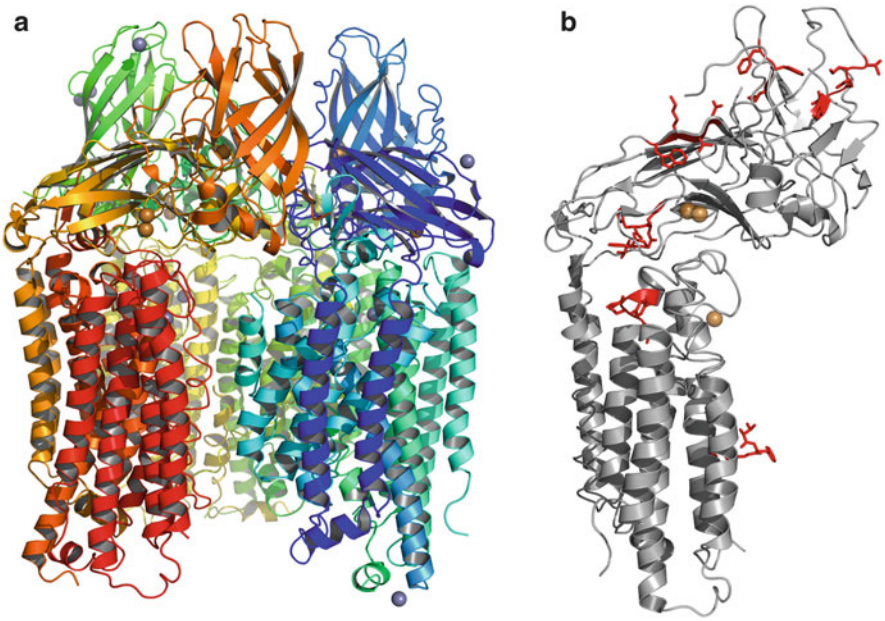


Fig. 11.9 Structure of pMMO from *M. capsulatus* BATH (PDB ID:1YEW). (**a**) Whole structure of pMMO (α3β3γ3). (**b**) Two copper sites are depicted in the orange ball in the one of the αβγ trimer

transfer from NADH through complex I, quinone pool, complex III, cytochrome c, and complex IV, and terminal electron acceptor is an oxygen. Many inhibitors are known for mitochondrial electron transfer chain. When rotenone or capsaicin was added to the membrane containing pMMO, NADH-dependent MMO activity decreased as shown in Fig. 11.10. On the contrary, complex III inhibitor, antimycin A, did not inhibit the NADH-dependent pMMO activity. These results indicate the existence of complex I in the cytoplasmic membrane containing pMMO. Complex I is an enzyme that transfers electrons from NADH to quinone pool in the membrane, so it is called as NADH:quinone oxidoreductase (NQO). NQO transfers electrons from NADH to quinone pool in the cytoplasmic membrane. The reduced form of quinone transfers electrons to the pMMO so that methane is oxidized to methanol by pMMO. When the rotenone was added to the membrane, NQO was partially inhibited. pMMO cannot accept electron from quinone pool so NADH-dependent MMO activity decreased. But in the case of duroquinol as an electron donor, duroquinol can transfer electron to pMMO directly, so duroquinol-dependent pMMO activity was observed in the presence of inhibitor.

NQO can be solubilized from membrane using same detergent as pMMO. After solubilization, purification was carried out using two anionic exchange chromatographies. The elution profile of NQO from Poros 20HQ column shows two NQO in the membrane. Both NQOs were inhibited by rotenone.

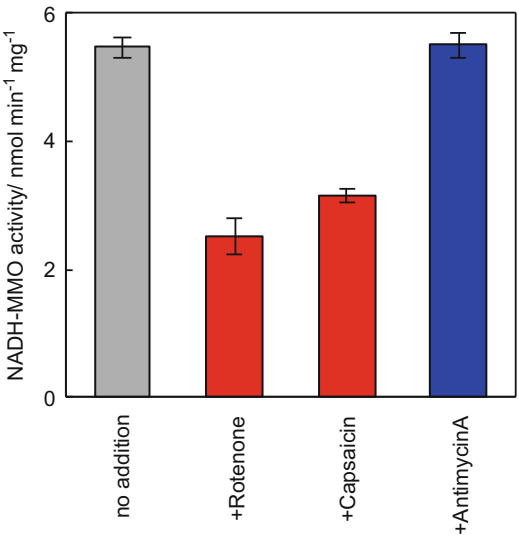


Fig. 11.10 Inhibition of pMMO containing membrane

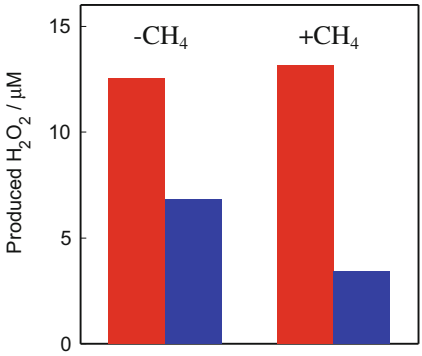


Fig. 11.11 Generation of hydrogen peroxide by membrane fraction in the presence of NADH. Red: after addition of NADH, Blue: incubate for 30 min with NADH

11.3.3 Inhibition of pMMO by Hydrogen Peroxide

It is known that the reactive oxygen species are generated from the mitochondrial electron transfer chain. Mitochondrial catalase protects cells from oxidative damages caused by hydrogen peroxide. When the pMMO activity in the membrane fraction was measured, pMMO activity increased by the addition of catalase, implying that hydrogen peroxide is generated by membrane containing pMMO with duroquinol, an electron donor for pMMO, and the generated H_2O_2 inhibits pMMO activity (Miyaji et al. 2009).To clarify the production hydrogen peroxide by the membrane fraction containing pMMO in the presence of electron donor, quantification of hydrogen peroxide was carried out. Figure 11.11 shows the generation of hydrogen peroxide by the membrane fraction containing pMMO in the presence of NADH as an electron donor. Hydrogen peroxide was generated simultaneously by the addition

of NADH to the membrane fraction in the presence or absence of methane. Production of hydrogen peroxide was also observed after 30 min incubation. These results indicate the generation of hydrogen peroxide by the electron transfer chain to pMMO and/or pMMO.

Figure 11.12 shows the effect of hydrogen peroxide to the EPR spectra of membrane fractions containing pMMO. Isolated membrane fraction showed typical type II EPR signal from pMMO, and intensity of this EPR signal increased by the addition of hydrogen peroxide. This indicates that hydrogen peroxide served as an oxidant for pMMO and copper ions were oxidized by hydrogen peroxide. In the presence of electron donor (reductant) such as NADH or duroquinol, copper ions in the pMMO were reduced and intensity of EPR signals decreased as shown in Fig. 11.7. In the presence of hydrogen peroxide, however, reduction of copper ions was not observed. These results suggest that hydrogen peroxide oxidizes copper ions and inhibits the activation of oxygen in the active site of pMMO. Low activity of purified pMMO is partially due to the inhibition by hydrogen peroxide generated by pMMO itself. To increase the activity of pMMO, regulation of electron transfer is important.

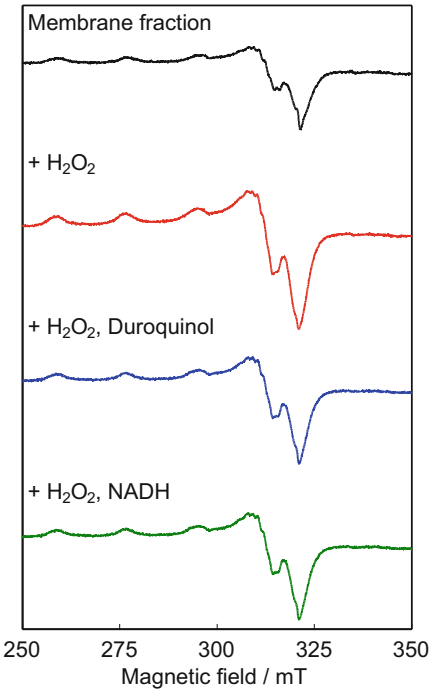


Fig. 11.12 Effect of hydrogen peroxide to the EPR spectra of membrane fractions containing pMMO

11.3.4 Methane Hydroxylation Using Light Energy by the Combination of thylakoid and MMO

MMO is a useful catalyst for methane oxidation and desirable application for methanol biosynthesis. One of the problems of application of MMO for biosynthesis of methanol is the source of electron donor. In the microorganism, electrons needed for oxidation of methane are supplied by the catabolic reaction of methane to carbon dioxide. To synthesize methanol from methane mentioned above, electrons donor such as formate is needed. One of the most promising candidates for the electron donor for methanol biosynthesis from methane is water. In photosynthesis, electrons are extracted from water to carry out the electron-transfer reaction in photosynthetic center to generate a proton motive force for synthesis of ATP and also production of physiological electron donor NAD(P)H. So electron donor can be generated by the combination of light energy and water. The outline of a photoinduced methane hydroxylation system by the combination of photosynthetic system and MMO was shown in Fig. 11.13.

Thylakoid membrane was obtained from spinach leaves, and membrane fraction containing pMMO was isolated from *M. trichosporium* OB3b. Photoinduced methane oxidation was carried out by the irradiation of light to the mixture containing chlorophyll from spinach and pMMO containing membrane fraction from *M. trichosporium* OB3b in the presence of NAD^+ as an electron carrier. Results are shown in Fig. 11.14. By the irradiation of light to the reaction mixture, the amount of methanol was increased. The amount of methanol obtained in the dark condition was apparently lower than that obtained under irradiation of light. These results indicate that selective oxidation of methane to methanol can be achieved by using electrons obtained from water oxidation by chlorophyll under irradiation of light.

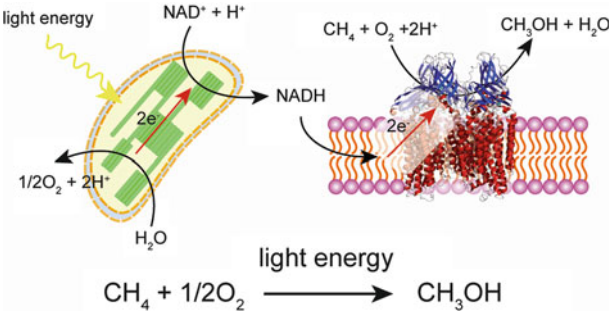


Fig. 11.13 Schematic diagram of photoinduced methane oxidation system using methane monooxygenase by the combination of photosynthetic system

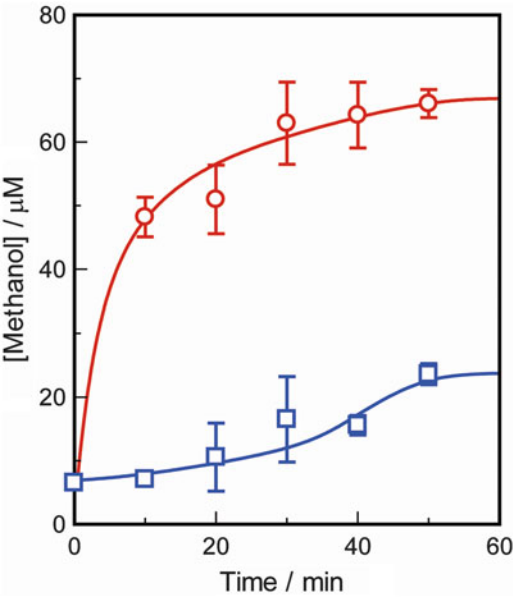


Fig. 11.14 Time dependence of methanol concentration in the reaction mixture containing NAD^+, thylakoid, membrane fraction from *M. trichosporium* OB3b, and methane with (red, open circle) or without (blue, open square) light irradiation

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The Biochemistry and Physiology of Respiratory-Driven Reversed Methanogenesis

12

Hadi Nazem-Bokaee, Zhen Yan, Costas D. Maranas, and James G. Ferry

12.1 Introduction

Methane is an important player in the global carbon cycle. Methanogenic microbes produce an estimated 1 gigaton (Gt) of methane annually of which most is reoxidized to CO_2 by aerobic and anaerobic microbes controlling release to the atmosphere of this potent greenhouse gas (Conrad 2009). The anaerobic oxidation of methane (AOM) in marine sediments alone is estimated to recycle greater than 70 billion kilograms of methane yearly (Reeburgh 1996). Apart from the environmental impact and abundant methane as an important fossil fuel, biomanufacturing processes are advocated for anaerobic conversion of methane to liquid biofuels and value-added products (Clomburg et al. 2017). Abating release to the atmosphere and advancing biotechnological applications are dependent on a mechanistic understanding of AOM.

AOM is initiated by anaerobic methanotrophic (ANME) species of the domain *Archaea* (Timmers et al. 2017). It is without question that ANME accomplish AOM by reversal of methanogenic pathways (Timmers et al. 2017). AOM requires the reduction of electron acceptors such as metal oxides, sulfate, nitrate, or nitrite to be thermodynamically favorable (Welte et al. 2016; Ettwig et al. 2016; Beal et al. 2009). Although ANME were first discovered in symbioses with sulfate-reducing species, it

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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_12

has since been inferred from enrichment and in situ studies that ANME alone are capable of respiratory-driven AOM by direct reduction of Fe(III), Mn(IV), nitrite, or nitrate (Welte et al. 2016; Ettwig et al. 2016; Beal et al. 2009; Oni and Friedrich 2017; Raghoebarsing et al. 2006; Hu et al. 2009). Three methanotrophic clades have been identified based on the 16S rRNA sequences of metagenomes (Timmers et al. 2017). The ANME-1 clade is related to the methanogen orders Methanomicrobiales and Methanosarcinales, whereas the ANME-3 clade is related to the genus *Methanococcoides* (Knittel et al. 2005; Hinrichs et al. 1999). The Methanomicrobiales and *Methanococcoides* are H_2-utilizing obligate CO_2-reducers, whereas the *Methanosarcina* are more metabolically diverse utilizing acetate and methylotrophic substrates (methanol, trimethylamines, and dimethyl sulfide) for methanogenesis and growth. Indeed, most *Methanosarcina* species are incapable of metabolizing H_2 (Guss et al. 2009). Evidence for respiratory-driven AOM is confined to the ANME-2 clade which is phylogenetically related to the order Methanosarcinales (Hinrichs and Boetius 2002). The Fe(III)- and nitrate-reducing *Candidatus* '*Methanoperedens nitroreducens*' (*Candidatus* '*Methanoperedenaceae*' fam. nov.) belongs to ANME-2d sub-clade (also named GOM Arc I and AOM-associated archaea) (Ettwig et al. 2016; Haroon et al. 2013; Mills et al. 2003; Lloyd et al. 2006). The Fe(III)-reducing *Methanosarcina acetivorans* is phylogenetically related to the ANME-2a sub-clade and is the only confirmed ANME in axenic culture (Soo et al. 2016; Nazem-Bokaee et al. 2016; Harder 1997; Moran et al. 2005, 2007). This review focuses on the biochemistry and physiology of respiratory-driven AOM. Key enzymes involved in reversal of methanogenesis and energy conservation are discussed. Finally, metabolic modeling of Fe(III)-dependent AOM by *M. acetivorans* is presented.

12.2 Nitrate Respiration

The axenic culture of a nitrate-reducing ANME has not been reported. Nonetheless, *Ca.* '*M. nitroreducens*' was named based on metabolic analysis of a reconstructed genome from the metagenome of a bioreactor showing simultaneous AOM and nitrate reduction (Haroon et al. 2013). Phylogenetic analyses based on 16S rRNA gene amplicon profiling placed *Ca.* '*M. nitroreducens*' in the ANME-2d sub-clade. A reverse CO_2-reduction pathway was postulated based on the finding of genes homologous to those of CO_2-reducing methanogens (Fig. 12.1). Metatranscriptome analyses showed high expression of the genes. Notably, expression of *mer* is contrary to its absence in the metagenomes of ANME-1 (Meyerdierks et al. 2010; Hallam et al. 2004). Genes (*narHG*) encoding nitrate reductase were identified in the genome of *Ca.* '*M. nitroreducens*,' although none for denitrification. Analyses suggest horizontal gene transfer of *narHG* from *Proteobacteria* of the domain *Bacteria*. The expression of *cdhBDEG* encoding acetyl-CoA synthase is consistent with acetate as a product of AOM by *Ca.* '*M. nitroreducens*.' Acetate is also a product of AOM by *M. acetivorans* and in sediments containing ANME-1 (Soo et al. 2016; Moran et al. 2007; Meyerdierks et al. 2010). Thus, acetate is a potential

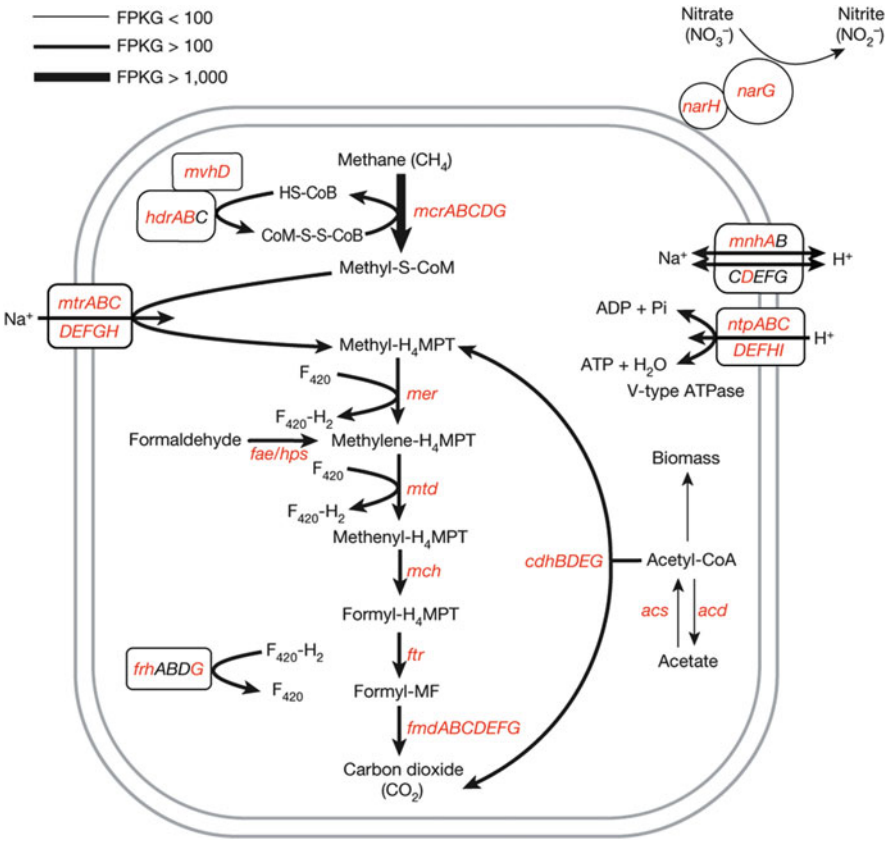

Fig. 12.1 Reverse methanogenesis pathway deduced from the reconstructed genome of *Candidatus* '*Methanoperedens nitroreducens*' ANME2D. Expressed genes determined by the metatranscriptomics are shown in red. Line thickness of reactions correlates with absolute expression. FPKG (fragments mapped per kilobase of gene length) is a measure of normalized gene expression. Genes and encoded enzymes: *mvhD*, subunit of the methyl viologen-reducing hydrogenase; *hdrABC*, cytoplasmic heterodisulfide reductase; *mcrABCDG*, methyl-coenzyme M methyl reductase; *mtrABCDEFGH*, methyl-H_4MPT:coenzyme M methyltransferase complex; *mer*, coenzyme F_{420}-dependent methylene-H_4MPT reductase; *mtd*, coenzyme F_{420}-dependent methylene-H_4MPT dehydrogenase; *mch*, cyclohydrolase; *ftr*, formyl-methanofuran (MF):H_4MPT, formyltransferase; *fmdABCDEFG*, formyl-MF dehydrogenase; *cdhBDEG*, CO dehydrogenase/acetyl-CoA synthase; *acs*, acetyl-CoA synthetase; *acd*, acetyl-coenzyme A synthetase (ADP forming); *ntpABCDEGHI*, V-type ATP synthase; *mnhABCDEFG*, multi-subunit sodium/proton antiporter. Reproduced by permission (Haroon et al. 2013)

intermediate in syntrophic marine AOM consortia comprised of ANME-2 and acetate-utilizing sulfate-reducing species. Syntrophic AOM is a symbiosis of anaerobic methanotrophic (ANME) archaea and sulfate-reducing species for which the latter utilizes reductant produced by the former that is overall thermodynamically favorable.

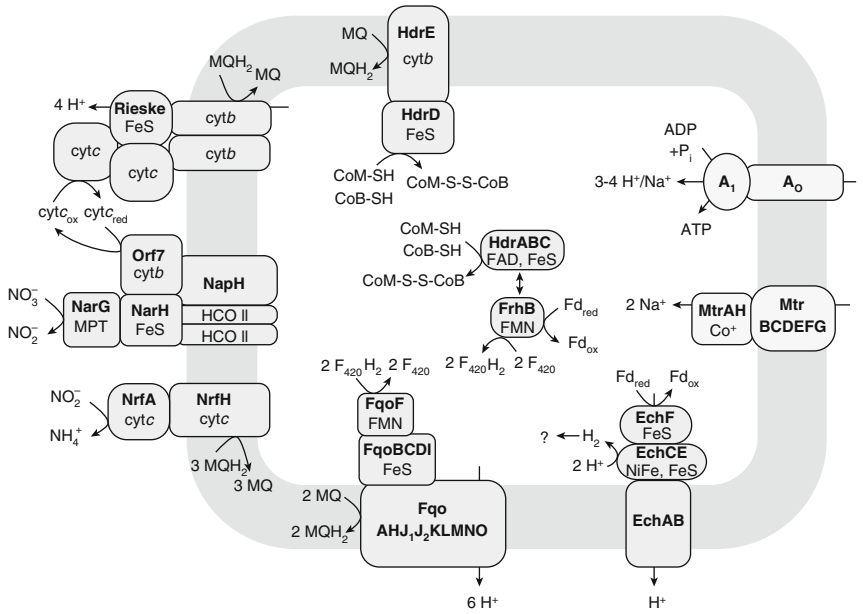

Fig. 12.2 Proposed model of membrane-bound electron transport in *Candidatus* '*Methanoperedens nitroreducens*' ANME2D. See text for explanations. Key: HCO II, heme copper oxidase subunit II-like proteins; cyt*b*, cytochrome *b*; cyt*c*, cytochrome *c*; FeS, iron-sulfur cluster; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; MPT, molybdopterin; NiFe, nickel-iron center; HdrABC, cytoplasmic heterodisulfide reductase; HdrDE, membrane-bound heterodisulfide reductase; Mtr, methyl-H_4MPT:coenzyme M methyltransferase complex; Fqo, coenzyme $F_{420}H_2$:quinone dehydrogenase; Nar, nitrate reductase; Nrf, nitrite reductase; Ech, hydrogenase; FrhB, F_{420}-reducing hydrogenase subunit B. Reproduced by permission (Arshad et al. 2015)

A second *Ca.* '*M. nitroreducens*' genome was reconstructed from the metagenome of another bioreactor coupling nitrate reduction with AOM (Arshad et al. 2015). The candidate organism was given the name *Ca.* '*M. nitroreducens*' MPEBLZ to distinguish it from the previous genome referred to as *Ca.* '*M. nitroreducens*' ANME2D. Comparison of the 16S rRNA gene sequences revealed 95% identity placing *Ca.* '*M. nitroreducens*' MPEBLZ within the ANME-2d sub-clade. A respiratory reverse methanogenesis pathway was postulated similar to that for *Ca.* '*M. nitroreducens*' ANME2D except with more details of proposed electron transport (Fig. 12.2) (Timmers et al. 2017; Arshad et al. 2015).

Analysis of *narHG* revealed an N-terminal TAT signal peptide for NarG consistent with transport outside the cytoplasmic membrane. The genome encodes homologs (Fqo) of the membrane-bound coenzyme $F_{420}H_2$ dehydrogenase complex of methylotrophic methanogens (Fpo) that generates a proton gradient (high outside the cytoplasmic membrane) coupled to oxidation of $F_{420}H_2$ and reduction of a quinone-like electron carrier called methanophenazine (Beifuss and Tietze 2005).

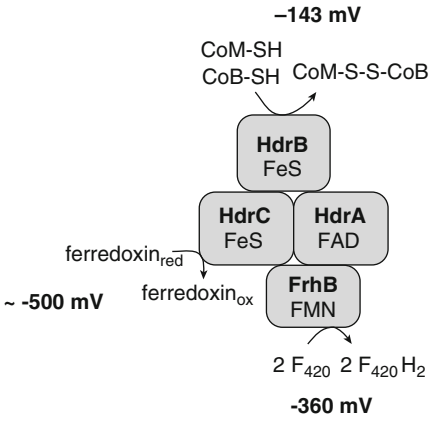

Fig. 12.3 Hypothesized cytoplasmic electron confurcating heterodisulfide reductase complex in *Candidatus* '*Methanoperedens nitroreducens*' ANME2D. Key: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FeS, iron-sulfur cluster; FrhB, F_{420}-reducing hydrogenase subunit B; HdrABC, heterodisulfide reductase subunits A, B and C. Reproduced by permission (Arshad et al. 2015)

Thus, it is proposed that Fqo oxidizes $F_{420}H_2$ generated in oxidation of methane to CO_2 (Fig. 12.2).

However, biochemical analysis of the bioreactor biomass revealed menaquinone (MQ)-like compounds to the exclusion of methanophenazine leading to the proposal that MQ replaces methanophenazine as the lipophilic electron carrier in reverse methanogenesis (Fig. 12.2). The genome of *Ca.* '*M. nitroreducens*' MPEBLZ encodes homologs of the membrane-bound heterodisulfide (CoMS-SCoB) reductase HdrED that functions in acetotrophic and methylotrophic *Methanosarcina* species. The HdrED homolog is postulated to regenerate the heterodisulfide needed for Mcr-catalyzed oxidation of methane utilizing MQ as the electron acceptor for oxidation of HSCoM and HSCoB generated in the Mcr- and Mtr-catalyzed reactions (Fig. 12.2).

The genome also encodes homologs of the cytoplasmic HdrABC heterodisulfide reductase HdrABC that functions in obligate CO_2-reducing methanogens. A role is proposed for the HdrABC homolog in regenerating CoMS-SCoB via an electron confurcating mechanism in which HdrABC oxidizes HSCoM and HSCoB and reduced ferredoxin generated in the last step of methane oxidation to CO_2 (Fig. 12.3). Electrons from HdrABC are passed to F_{420} via a homolog of FrhB from methylotrophic methanogens found encoded in the *Ca.* '*M. nitroreducens*' MPEBLZ genome. The exergonic reduction of F_{420} with reduced ferredoxin is coupled to the endergonic oxidation of CoM-SH/CoB-SH and reduction of F_{420}. However, the genome also encodes the homolog of a novel heterodisulfide reductase HdrA2B2C2 from *M. acetivorans* that bifurcates electrons from oxidation of $F_{420}H_2$ into ferredoxin and CoMS-SCoB where it is proposed to function in the Fe(III)-dependent AOM pathway (Yan et al. 2017). Thus, it is possible the HdrA2B2C2

homolog in *Ca.* '*M. nitroreducens*' MPEBLZ catalyzes the confurcation reaction without participation of FrhB. Other mechanisms for oxidation of reduced ferredoxin include the sodium-pumping Rnf complex common in the Methanosarcinales and Ech hydrogenase (Li et al. 2006; Suharti et al. 2014; Schlegel et al. 2012a; Welte et al. 2010). Although expression levels were low, only genes encoding the Ech hydrogenase were found in the genome consistent with H_2 as a product of AOM although the fate of H_2 is unknown. The reduced MQ (MQH_2) generated by HdrED and Fqo is reoxidized via an unusual Rieske/cytochrome *b* complex encoded in the genome with homology to bc_1 complexes from the domain *Bacteria* that couple oxidation of quinones to reduction of cytochrome *c* with generation of a proton gradient via the Q cycle. Transcriptome analyses show genes encoding *c*-type cytochromes encoded adjacent to the Rieske/cytochrome *b* complex, a result consistent with electron transfer from MQH_2 to NarGH and reduction of nitrate (Fig. 12.2). Genes encoding acetate kinase or nucleotide phosphate-dependent acetyl-CoA synthetase are absent in the *Ca.* '*M. nitroreducens*' MPEBLZ genome ruling out substrate-level phosphorylation. Instead, the genome encodes an ATP synthase driven by H^+ or Na^+ gradients. Proton gradients are generated by the Fqo and the Rieske/cytochrome *b* complexes. Thus, it would appear that a H^+/Na^+ antiporter is required for generation of the Na^+ gradient required for ATP synthesis and driving the Na^+-dependent endergonic methyl transfer catalyzed by CH_3-CoM: tetrahydromethanopterin (H_4MPT) methyltransferase (Mtr) (Fig. 12.2). The reconstructed genome of *Ca.* '*M. nitroreducens*' ANME2D contains genes encoding a homolog of the multi-subunit H^+/Na^+ antiporter (MnhA-G) that may fulfill this function (Fig. 12.1).

Nitrite was reduced to ammonium in the bioreactor from which the *Ca.* '*M. nitroreducens*' MPEBLZ genome was reconstructed (Arshad et al. 2015). Thus, it is of interest that the *Ca.* '*M. nitroreducens*' MPEBLZ genome contains genes encoding the NrfAH-type cytochrome *c* nitrite reductase homologous to those of the δ- and ε-*Proteobacteria*. The catalytic subunit of NrfA contains a signal peptide consistent with translocation to outside the cytoplasmic membrane (Fig. 12.2). It should be noted here that the anaerobe *Methylomirabilis oxyfera* has been shown responsible for nitrite-dependent AOM albeit by a different mechanism. Prior to reduction of nitrite to NO, the organism dismutates NO to N_2 and O_2 with use of the O_2 for activation of methane by methane monooxygenase (Ettwig et al. 2010, 2012).

12.3 Fe(III) Respiration

12.3.1 Metagenomic Pathway

Studies indicate that marine sediments contain ANME from the ANME-2a sub-clade that directly reduce insoluble forms of Fe(III) with the potential for independent respiratory growth (Scheller et al. 2016; McGlynn et al. 2015). A genome was reconstructed from the metagenome of a syntrophic sulfate-reducing AOM

enrichment culture derived from a marine environment populated with ANME-2a (Wang et al. 2014). The 16s rRNA sequence revealed that the genome belongs to an unnamed species from the ANME-2a sub-clade. Further, all genes in the reconstructed genome were taxonomically assigned to the order Methanosarcinales where phylogenetic analyses predict ANME-2a species reside. A postulated reverse methanogenesis AOM pathway was deduced from the reconstructed genome containing homologous genes encoding enzymes of methanogenic pathways (Fig. 12.4). The ANME-2a pathway is similar to the nitrate-reducing AOM pathways of the ANME-2d sub-clade with notable exceptions for electron transport. Foremost, enzymes and electron carriers predicted for nitrate and nitrite reduction in the ANME-2d pathways of *Ca.* '*M. nitroreducens*' are absent in the ANME-2a pathway. Further, a cytoplasmic HdrABC family heterodisulfide reductase, the Rnf complex, and methanophenazine play prominent roles in the ANME-2a pathway. The Rnf complex pumps Na^+ that drives the endergonic Mtr-catalyzed methyl transfer. The H^+ gradient generated by Fpo presumably drives ATP synthesis by an ATP synthase. Finally, multi-heme *c*-type cytochromes (MHC) are proposed to donate electrons to Fe(III) or bacterial partners in syntrophic AOM by transferring electrons from methanophenazine through the S-layer outer envelope (McGlynn et al. 2015). A role for MHCs is also likely for Fe(III)-dependent AOM by *Ca.* '*M. nitroreducens*' (Ettwig et al. 2016). Importantly, hydrogenases are absent in the reconstructed genome suggesting interspecies H_2 transfer fails to play a role in syntrophic AOM of environments dependent on ANME-2a. However, the potential for acetate as a product of ANME-2a suggests a role in syntrophic AOM involving acetate-utilizing sulfate-reducing species.

12.3.2 Methanosarcina Acetivorans Pathway

The axenic culture of an ANME has not been reported; however, the marine archaeon *M. acetivorans* is capable of methane oxidation during methanogenesis ("trace methane oxidation") and methanotrophic growth dependent on reduction of Fe(III) (Soo et al. 2016; Nazem-Bokaee et al. 2016; Moran et al. 2005, 2007). The ability to reduce Fe(III) is not surprising in view of reports that *Methanosarcina* species reduce soluble and insoluble oxides of Fe(III) during methanogenesis (Sivan et al. 2016; Bond and Lovley 2002). *M. acetivorans* produces all the enzymes of the reconstructed ANME-2a pathway (Fig. 12.4) derived from the reconstructed genome of an uncultured marine ANME species phylogenetically related to *M. acetivorans* (Orphan et al. 2001). This section reviews the biochemical-based Fe(III)-dependent respiratory AOM pathway proposed for *M. acetivorans* and presents a metabolic model based on the pathway.

Pathway The pathway (Fig. 12.5) is primarily a reversal of the acetate-utilizing methanogenic pathway (reactions 1–5, 7, 8, 16, 18, 19) of *M. acetivorans* (Ferry 2015). Reactions 1 and 2 are endergonic requiring oxidation of HSCoM and HSCoB coupled to the exergonic reduction of Fe(III) (reactions 1–6). Proton translocation is

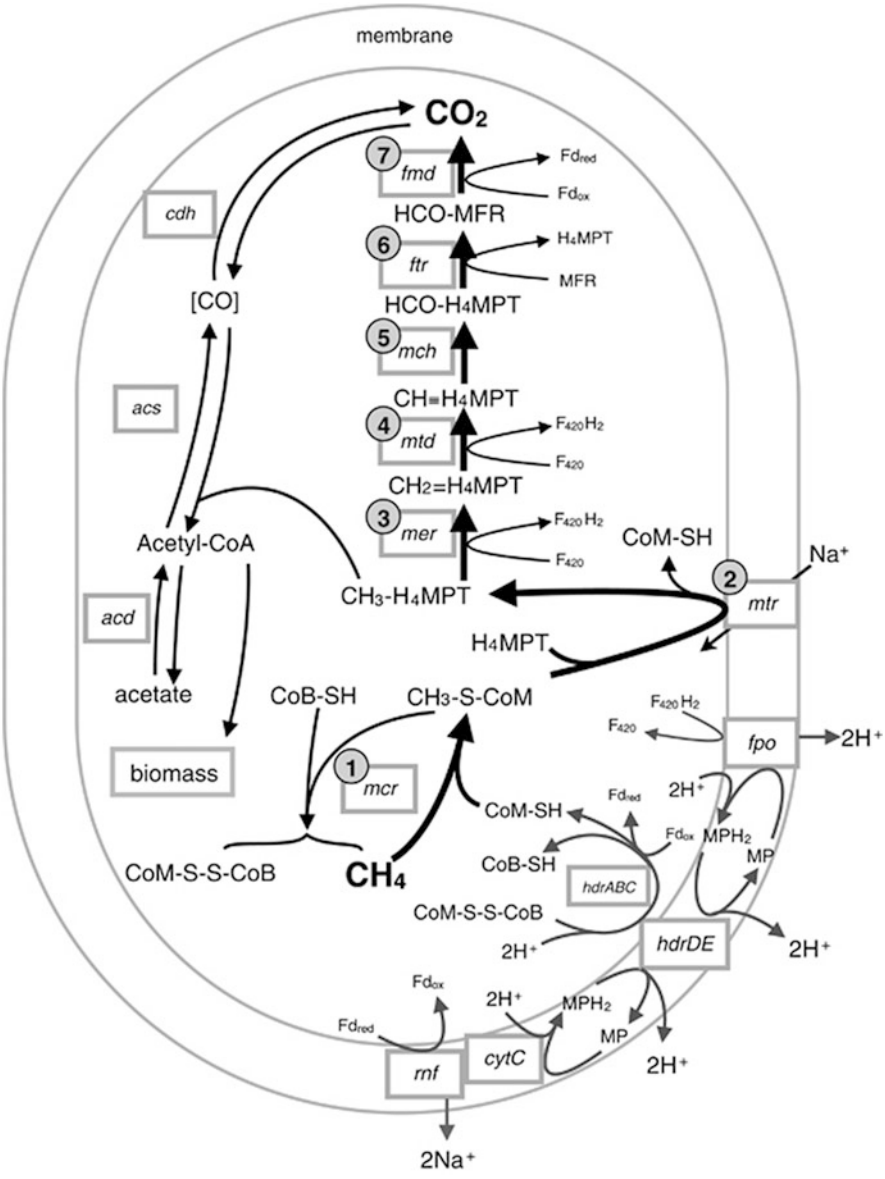

Fig. 12.4 Proposed AOM pathway deduced from the reconstructed genome of the ANME-2a sub-clade. Positive gene identifications are displayed in boxes. Carbon transformations of the reverse methanogenic pathway are shown with thick black arrows and electron transport with thinner gray arrows. Gene designations are as in Fig. 12.1 with the following additions: *rnf*, electron transport complex; *fpo*, coenzyme $F_{420}H_2$ dehydrogenase; *cytC*, multi-heme cytochrome *c*; MP, methanophenazine. Reproduced by permission (Sivan et al. 2016)

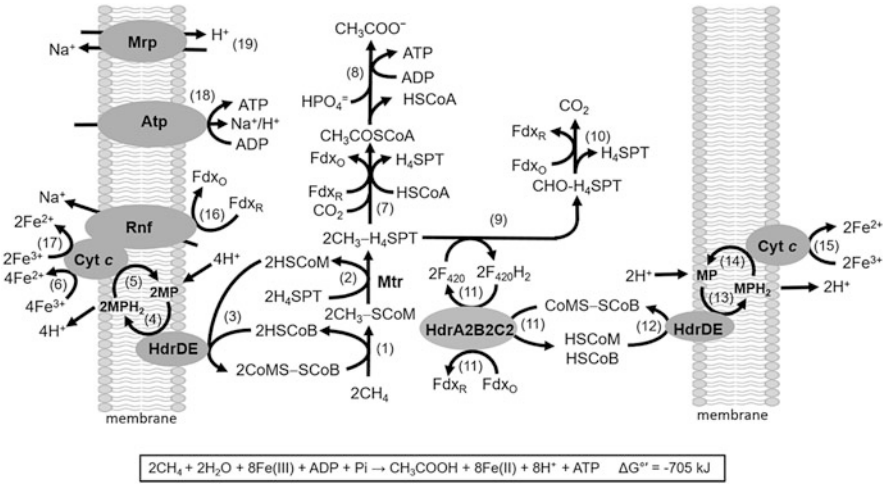

Fig. 12.5 Pathway proposed for Fe(III)-dependent anaerobic oxidation of methane by *M. acetivorans*. Reaction numbers corresponding to the text are shown in parentheses. Key: CoA-SH, coenzyme A; H_4SPT, tetrahydrosarcinapterin; Fdx_R, reduced ferredoxin; Fdx_O, oxidized ferredoxin; HSCoM, coenzyme M; HSCoB, coenzyme B; MP, methanophenazine; HdrDE, membrane-bound heterodisulfide reductase; HdrA2B2C2, cytoplasmic heterodisulfide reductase; Rnf, electron transfer complex; Cyt*c*, cytochrome *c*; Atp, ATP synthase; Mrp, multi-subunit sodium/proton antiporter; Mtr, membrane-bound methyltransferase. Reproduced by permission (Yan et al. 2017)

accomplished by a "Q loop" mechanism involving methanophenazine (MP). Acetate-grown *M. acetivorans* is rich in MHCs that shuttle electrons between the Rnf complex and MP (Li et al. 2006; Wang et al. 2011; Kletzin et al. 2015). Reduction of Fe(III) at MHCs is consistent with proposed roles for electron transfer to Fe(III) and bacterial partners in syntrophic AOM (Timmers et al. 2017; Scheller et al. 2016; McGlynn et al. 2015).

The methyl group of CH_3-H_4SPT (reaction 2) is either incorporated in acetate or oxidized to CO_2. The formation of acetate begins with reaction 7 requiring reduced Fdx for reduction of CO_2 that becomes the carbonyl group of acetyl-CoA. The Fdx is reduced on oxidation of the methyl group to CO_2 (reaction 10) that also involves two oxidations (reaction 9) dependent on F_{420} (Deppenmeier, 2004). The $F_{420}H_2$ is oxidized by HdrA2B2C2 that bifurcates electrons from $F_{420}H_2$ into Fdx and CoMS-SCoB (reaction 11). The bifurcation is coupled to reactions 12–15 reducing Fe(III) with an overall $\Delta G^{\circ\prime}$ of −187.1 kJ. Reactions 12–15 are catalyzed by the same proteins and electron carriers as for reactions 3–6. A proton gradient (high outside) is generated by a "Q loop" mechanism involving MP. The exergonic oxidation of Fdx and reduction of Fe(III) in reactions 16 and 17 are coupled to generation of a Na^+ gradient consistent with that reported for reduction of cytochrome *c* and pumping of Na^+ by Rnf (Schlegel et al. 2012a; Wang et al. 2011). Notably, an Rnf homolog is encoded in the *Ca.* '*M. nitroreducens*' ANME2A reconstructed genome consistent with an important role in AOM pathways for

ANME of the ANME-2a sub-clade (Fig. 12.4) (Wang et al. 2014). The endergonic Mtr-catalyzed reaction 2 is driven by the Na^+ gradient generated by Rnf. ATP synthesis is catalyzed by the Na^+- and H^+-dependent ATP synthase (reaction 18) (Schlegel et al. 2012b). ATP is also synthesized by substrate-level phosphorylation (reaction 8) catalyzed by phosphotransacetylase and acetate kinase (Aceti and Ferry 1988; Lundie and Ferry 1989). A multi-subunit Na^+/H^+ antiporter (Mrp) adjusts the ratio of Na^+/H^+ (reaction 19) optimal for the Na^+- and H^+-dependent ATP synthase (Jasso-Chavez et al. 2013, 2016). A role for multi-subunit Na^+/H^+ antiporters in AOM is supported by a homolog of a multi-subunit H^+/Na^+ antiporter (MnhA-G) encoded in the reconstructed genome of *Ca.* '*M. nitroreducens*' ANME2D (Fig. 12.1).

The stoichiometry shown in Fig. 12.5 assumes a low availability of Fe(III) that limits the oxidation of Fdx by reactions 16 and 17 that becomes available for the Fdx-dependent synthesis of acetate (reactions 7 and 8). Thus, when Fe(III) is limiting, syntrophic AOM with an acetate-utilizing sulfate-reducing partner may dominate respiratory-driven AOM. Reactions 16 and 17 are more thermodynamically favorable than reactions 7 and 8; therefore, greater oxidation of methane to CO_2 would be expected when Fe(III) is non-limiting. The theoretical extent at which Fe(III) becomes limiting can be estimated through reconstructing the pathway shown in Fig. 12.5 as a metabolic network model.

Metabolic model Among genome-scale metabolic models available for the domain *Archaea*, there has been considerable effort on model development for *M. acetivorans* due to its versatile substrate utilization capabilities and the progress in its genetic manipulation (Thor et al. 2017). After the independent development of iVS941 (Satish Kumar et al. 2011) and iMB745 (Benedict et al. 2012) metabolic models for *M. acetivorans*, the iMAC868 model (Nazem-Bokaee et al. 2016) emerged as an update to these models by combining them and corrected numerous mass and charge imbalances, revised 64 GPRs based on most recent gene annotation data for over 700 genes, and appended necessary reactions which allowed for thermodynamically feasible reversal of the methanogenesis pathway. The iMAC868 metabolic model of *M. acetivorans* provides, for the first time, a computational platform for analyzing methane utilization in silico by *M. acetivorans* to capture metabolic phenotypes of the organism and allows for predicting theoretical limits of methane utilization, external electron acceptor usage, and production yields of potential biorenewable/biofuel precursors (Nazem-Bokaee et al. 2016). The recent experimental evidence on the expression of soluble heterodisulfide (HdrA2B2C2) as the sole electron bifurcation node in *M. acetivorans* utilizing methane (Yan et al. 2017) sheds light on our understanding of the electron flow mechanisms during Fe(III)-dependent AOM by this organism.

As expected, the iMAC868 model correctly failed to predict any methane utilization in the absence of ferric or any other electron acceptor. Starting with an arbitrary fixed uptake rate of 10 mmol methane per gram dry cell weight per hour (mmol/gDCW/h), a minimum Fe(III) reduction rate of 5 mmol/gDCW/h is essential to drive the reversal of methanogenesis pathway (Fig. 12.6). As Fe(III) availability increases,

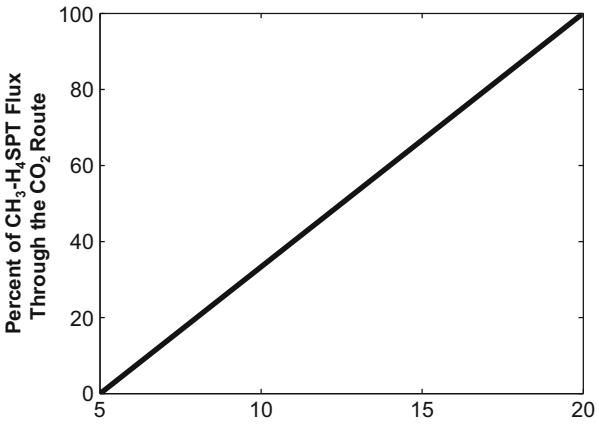

Fig. 12.6 Percentage of total CH_3-H_4SPT flux through the CO_2 pathway as a function of Fe(III) reduction rate predicted by the iMAC868 model of *M. acetivorans*

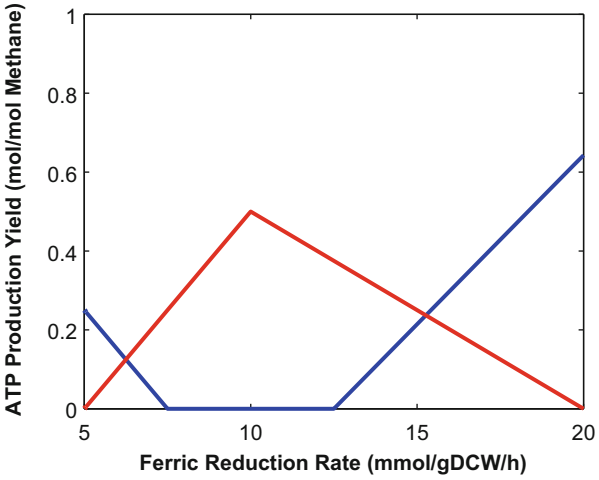

Fig. 12.7 ATP synthesis during AOM by *M. acetivorans* can switch between the chemiosmotic (blue) and substrate-level phosphorylation (red) routes. Production of ATP per mol of methane is shown as a function of Fe(III) reduction rate for in silico *M. acetivorans* predicted by the iMAC868 model

more CH_3-H_4SPT needs to be oxidized to CO_2 producing enough reducing equivalents required for shuttling electrons coming from methane to the external electron acceptor, i.e., Fe(III). The requirement of reduced Fdx and $F_{420}H_2$ as intermediate electron donors increases carbon flux through the methylotrophic pathway due to which methane availability for acetyl-CoA production is reduced. In the context of the model, it was predicted that at Fe(III) reduction rate of 10 mmol/gDCW/h, at least one third of CH_3-H_4SPT has to be oxidized to CO_2. At Fe(III) reduction rate of 20 mmol/gDCW/h, all methane has to be oxidized to CO_2, and beyond that, no methane can be utilized (Fig. 12.6).

When Fe(III) is limiting (i.e., at a rate of 5 mmol/gDCW/h), ATP is synthesized by ATP synthase only (Fig. 12.7) through chemiosmotic ATP synthesis route. As Fe (III) reduction rate increases, ATP production via substrate-level phosphorylation

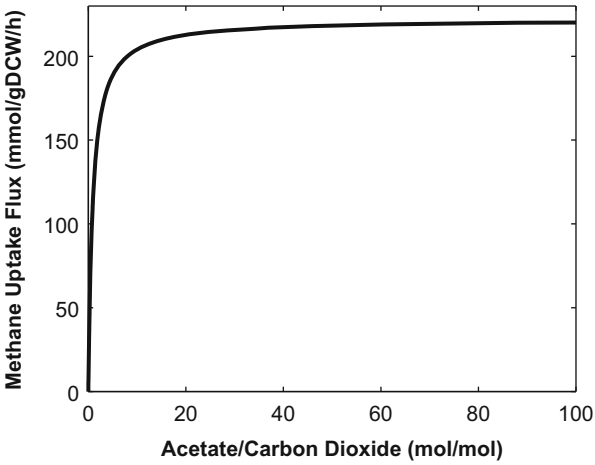

Fig. 12.8 Maximum theoretical ratio of acetate to carbon dioxide production as a function of methane utilization when unlimited source of Fe(III) is available as external electron acceptor predicted by the iMAC868 model of *M. acetivorans*

dominates as a result of which acetate production increases and reaches to its maximum at a Fe(III) reduction rate of 10 mmol/gDCW/h (Fig. 12.7). However, beyond this Fe(III) reduction rate, acetate production drops due to the shortage of reduced Fdx. The partitioning of CH_3-H_4SPT toward acetate production pathway (through reaction 7 in Fig. 12.5) drops to 50% owing to the increasing mandatory flux through the methylotrophic pathway to generate reducing equivalents needed for transferring electrons to the increased levels of Fe(III). At this point, ATP synthesis via chemiosmotic route switches on, and when the flux of CH_3-H_4SPT through methylotrophic pathway passes 68.5%, the chemiosmotic ATP production dominates again (Fig. 12.7). At Fe(III) reduction rate of 20 mmol/gDCW/h, ATP is only produced by ATP synthase (Fig. 12.7).

If there is an unlimited pool of Fe(III) (i.e., methane uptake not fixed), and if the in silico *M. acetivorans* cell can release as much CO_2 as needed during AOM, by fixing the acetate production rate and solving for the rate of CO_2 excretion, it is found that the portion of methane ending up in acetate can be as high as 95% of that oxidized to CO_2 (Fig. 12.8). This is provided that equi-molar Fe(III) and methane exist at all times during AOM.

12.4 Outlook

In situ ecological and metagenomic investigations have provided a wealth of information that sets the stage for a mechanistic understanding of AOM. However, the isolation of pure cultures from a variety of AOM environments is paramount to reach this next level of understanding. The finding that ANME of the ANME-2 clade have the capacity for respiratory growth will undoubtedly facilitate isolations needed to advance a mechanistic understanding. In particular, roles are indicated for electron bifurcation and confurcation by HdrABC family enzymes and the Rnf complex which appears to be a universal requirement for energy conservation. Finally, the robust genetic system of *M. acetivorans* holds still greater promise for significant contributions.

Acknowledgments Research in the laboratories of CDM and JGF was supported by the US Department of Energy ARPA-e grant 0881-1525.

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Methylotrophic Cell Factory as a Feasible Route for Production of High-Value Chemicals from Methanol

13

Lanyu Cui, Chong Zhang, and Xin-Hui Xing

13.1 Introduction

Methanol is an important building block in the chemical industry. As shown in Fig. 13.1, it can be synthesized from both fossil fuels and renewable raw materials such as waste biomass and CO_2 (Bertau et al. 2014). Methanol can also be synthesized from methane in one step over a nickel catalyst at 800 °C−1000 °C and 5−40 bar of pressure in a process known as bi-reforming (Bertau et al. 2014), and that can be represented by the following formula:

$$3CH_4 + CO_2 + 2H_2O \rightarrow 4CO + 8H_2 \rightarrow 4CH_3OH$$

Abundant methane supplies from diverse chemical or biochemical pathways make methane a suitable substance for biosynthesis (Zhang et al. 2008; Kalyuzhnaya et al. 2015). This process has been proposed for all kinds of natural gas and even shale gas. Catalysts able to activate methane at room temperature (Zuo et al. 2016) and with higher product selectivity (Mahyuddin et al. 2016) are being investigated. What's more, methanol can also be biologically produced from methane, which can be produced by anaerobic fermentation of the renewable biomass resources. The enzyme of methane monooxygenase (MMO) catalyses the conversion of methane to methanol (Blanchette et al. 2016), and it could therefore

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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_13

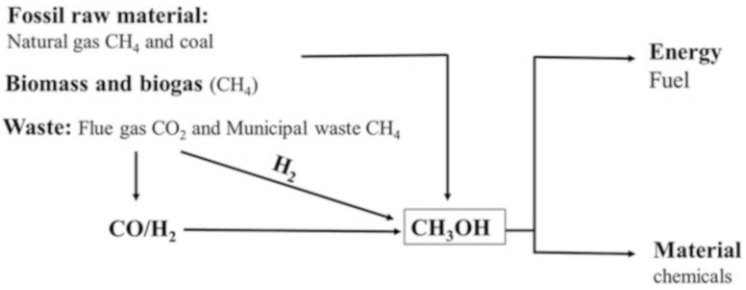

Fig. 13.1 Methanol production pathways

contribute to the methanol-based economy that has been discussed widely in the scientific community (Olah et al. 2009).

Most microbial cell factories are based on glucose or sugar as the feedstocks, which causes two problems. Sugars are usually derived from grain and thus would compete with human food sources, resulting in the higher costs. The second is the technical and economic bottleneck for bioproduction using lignocellulose as the feedstock. In contrast, methanol is now produced at overcapacity, especially after the shale gas revolution, which is used as a microbial carbon source worldwide. Methanol-based biosynthesis is one of the most well-studied and most promising C1 metabolisms for MeCFs.

Biotechnology based on methanol-utilizing bacteria has been well demonstrated by large-scale single-cell protein production. In recent years, our understanding of the metabolism of these bacteria and the toolkits for their genetic engineering have been expanded, resulting in new strategies for production of fine and bulk chemicals as well as biofuels by methylotrophic bacteria as the MeCFs.

13.2 Methanol-Utilizing Bacteria

Methanol-utilizing bacteria are able to utilize methanol and some other single carbon compounds, but not methane, as a sole carbon and energy source. They include a variety of Gram-positive and Gram-negative species. Based on the range of carbon compounds utilized, methanol-utilizing bacteria can be divided into three groups, as shown in Fig. 13.2. Some researchers have introduced methanol metabolic pathways into *Escherichia coli* to construct artificial methanol-utilizing bacteria (Peyraud et al. 2011). However, the yield of the target product remains quite low because five-carbon sugar supplementation remains a problem and the tolerance of the engineered *E. coli* strain to methanol is poor, while a methanol-based MeCF using methylotrophic bacteria is likely to be more efficient and feasible as the direct bioproduction approaches.

Over the past five decades, the α-proteobacterium *Methylobacterium extorquens* has been investigated both physiologically and biochemically. Using complementary omic technologies such as transcriptomics, proteomics, metabolomics and

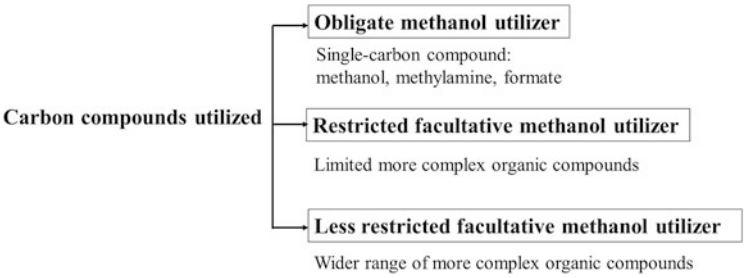

Fig. 13.2 Methanol assimilation pathways in *Methylobacteria* (Michelsson and Linen 1987; Jenkins and Jones 1987)

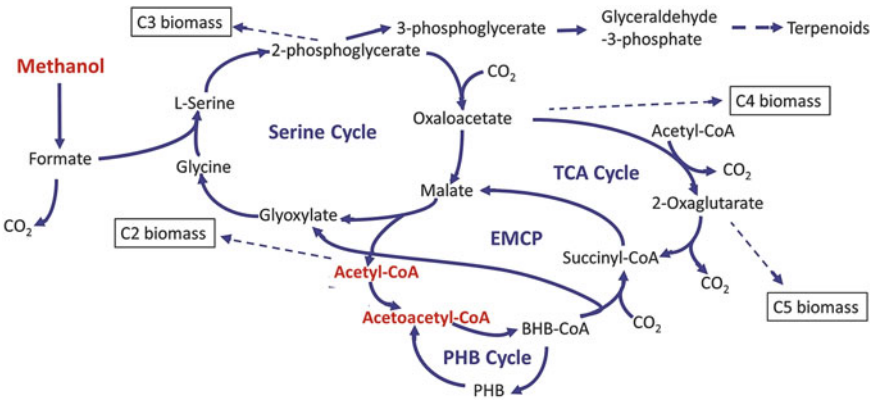

Fig. 13.3 Metabolic pathways in AM1 grown on methanol (Zhu et al. 2016)

fluxomics, knowledge about *M. extorquens* AM1 has grown, and it is now used as a model methylotroph that is widely used to elucidate methanol metabolism and biosynthesis of the derived chemicals.

In 2011, Peyraud et al. (2011) used ^{13}C flux-based methods to describe metabolic network patterns in AM1 and measured the carbon flux distribution of the central metabolic pathway. Methanol was oxidized into aldehydes and then converted into formic acid, among which 84% was dissimilated into carbon dioxide to generate NADH and 16% entered into the assimilation pathway. As shown in Fig. 13.3, pathways for AM1 growth based on methanol as the substrate include the methanol oxidation pathway, the one-carbon transfer pathway, the formic acid oxidation pathway, the tricarboxylic acid (TCA) cycle and the ethylmalonyl-CoA (EMC) cycle.

In the methanol oxidation pathway, the methanol is oxidized in the periplasmic space. Methanol dehydrogenase (MDH) is a key $\alpha_2\beta_2$ tetrameric enzyme in which the two subunits are encoded by *mxaF* and *mxaI*, respectively. In addition to MDH, AM1 also contains another methanol dehydrogenase XoxF (Schmidt et al. 2010) that not only catalyses the oxidation of methanol but also formaldehyde and ethanol.

AM1 contains two sets of methanol dehydrogenases, with one acting in the one-carbon transfer pathway in which methanol is oxidized to aldehydes in the periplasmic space and then transported into cells. This pathway mediates the cellular detoxification. Meanwhile, the formic acid oxidation pathway involves dehydrogenase-based oxidation into two carbon units and NADH. The serine cycle lies at the centre of metabolism in this bacterium and accounts for the majority of the carbon flow, and glyoxylic acid is transformed into glycine to feed back into the serine cycle. Acyl-CoA is fed into the EMC cycle that acts as a CoA derivatives conversion pathway, generating glyoxylic acid that feeds back into the serine cycle. Two acetyl-CoA molecules are condensed in six steps to produce methylmalonyl-CoA, which is then split into glyoxylic acid and propionyl-CoA. Most of the enzymes in the EMC cycle are shared with other metabolic pathways, while ethylmalonyl-CoA mutase (Ecm) (Erb et al. 2008) and crotonyl-CoA reductase and carboxylase (Ccr) (Erb et al. 2007) are unique to this pathway. Acetyl-CoA is the key intermediate in the EMC pathway, and it can be converted into other dicarboxylic acids or fatty acids.

As shown in Fig. 13.3, the serine cycle, TCA cycle and EMC cycle combine together to form a complex metabolic network that is crucial for allowing AM1 to grow on methanol. However, the exact regulation mechanism for the metabolic flux in this strain still remains unknown. Some regulators have been reported in AM1 and could conceivably be used for directing carbon flux into the target products.

13.3 Metabolic Regulators in AM1

The central metabolic pathways operating in AM1 are complex, and the regulatory mechanisms are unclear. Several regulatory factors have been reported that may contribute to metabolic regulation (Table 13.1).

The metabolic flux of the TCA pathway in a PhaR knockout mutant was evaluated by ^{13}C labelling (Van Dien et al. 2003). The metabolic flux of the EMC pathway was found to be weakened, and no acetyl-CoA was introduced into the PHB pathway, suggesting that the role of PhaR is to control the distribution of acetyl-coA in the EMC and TCA pathways.

Table 13.1 Regulators reported in AM1

Regulator	Function	References
PhaR	Regulate acetyl-CoA metabolism	Van Dien et al. (2003)
QscR	Serine cycle pathway and carbon transfer pathway	Van Dien et al. (2003) and Zhu et al. (2016)
PhyR	Stress response factor, positive transcriptional regulation of 246 genes	Gourion et al. (2008)
MtdA	May regulate formic acid metabolism	Skovran et al. (2010)
CcrR	Regulation of the expression of the key enzyme Ccr in the EMC pathway	Hu and Lidstrom (2012)

QscR is perhaps the most well-studied regulator in AM1 (Kalyuzhnaya and Lidstrom 2003, 2005). QscR is a transcriptional regulator of the serine cycle that binds to the promoter region and induces the expression of three gene clusters, namely, the *sga-hpr-mtdA-fch* gene cluster, the *mtkA-mtkB-ppc-mclA* gene cluster and the *glyA* gene. It also induces its own expression. Glyoxylic acid, CoA and $NADP^+$ inhibit the regulation by QscR, while formyltetrahydrofolate strengthens the regulation. Valdez and Skovran (2014) found that QscR also affects the expression of formate dehydrogenase, and it is therefore also a formic acid metabolism control point. A high formic acid concentration releases the self-repression of QscR.

PhyR is a stress-response regulator (Gourion et al. 2008) consisting of two domains, a sigma factor-binding domain at the amino-terminus and a receptor domain at the C-terminus. Its action mechanism has been confirmed (Francez-Charlot et al. 2009), and it has been proposed as a global transcriptional regulator functioning in response to heat shock, radiation, drought, salt tolerance and ethanol. Its role is proposed to be similar to the pressure response factors of *Rpo*S and *Rpo*B in *E. coli* (Metzger et al. 2013).

Methylenetetrahydrofolate deaminase (MtdA) catalyses the conversion of methane-tetrahydrofolate into methylenetetrahydrofolate in the last step of the carbon transfer pathway, and it may be regulated by a dedicated regulatory protein. Skovran et al. observed the intracellular changes when transferring AM1 from growth on succinate to methanol (Skovran et al. 2010). The initial level of MtdA transcription was high, but the enzyme was inactive, suggesting it might be post-transcriptionally regulated. When MtdA enzyme activity is increased, formic acid is decreased, indicating that MtdA transcriptional regulation might control formic acid metabolism.

The CcrR regulator acts on the EMC pathway and regulates the expression of the Ccr enzyme by binding to the *ccr* promoter without affecting other enzymes in the EMC pathway (Hu and Lidstrom 2012). Ccr is a key enzyme in the EMC pathway, and its regulation by CcrR is critical.

The nontargeted global transcriptional machinery engineering (gTME) (Alper et al. 2006) strategy has been used to successfully reprogramme the cellular transcriptome by engineering transcriptional regulators (Klein-Marcuschamer and Stephanopoulos 2008; Santos et al. 2012). Liang et al. (2017) demonstrated a new strategy for sensor-assisted transcriptional regulator engineering (SATRE) to control metabolic flux redistribution to increase acetyl-CoA flux from methanol to mevalonate synthesis in engineered AM1. QscR was used to redirect the metabolic flux and increase the acetyl-CoA supply for downstream synthesis of the metabolites. ^{13}C labelling revealed that acetyl-CoA flux was improved by 7%, and transcriptional analysis revealed that QscR had global effects at two key points, NADPH generation and *fum*C overexpression, both of which might contribute to carbon flux redistribution. A fed-batch fermentation of the QscR-49 mutant in a 5 L bioreactor yielded a mevalonate concentration of 2.67 g/L produced from methanol.

13.4 Production of Value-Added Chemicals by AM1

Methylobacteria are able to link inorganic and organic synthesis and are therefore well suited to future biochemical engineering applications for the production of single-cell protein, polyhydroxyalkanoates (PHAs) and amino acids. In AM1, PHAs are the best characterized and can be widely used in packaging bags, medicines, textiles and household materials. Recently, many studies have focused on production of dicarboxylic acids, biofuels and terpenes by AM1, as shown in Table 13.2.

AM1 can synthesize polyhydroxybutyrate (PHB) via the PHB cycle as an energy storage form in the cells. PHB production in AM1 has been studied for more than 30 years, and in 1986 Suzuki et al. increased the PHB titre to 149 g/L (Suzuki et al. 1986). Due to the limited rigidity and brittleness of the PHB structure, researchers have attempted to improve the mechanical properties using a multi-monomer copolymer approach, by adding pentanoic acid or amyl alcohol into the medium (Ueda et al. 1992). However, exogenous additives can increase the cost. In 2014, advances in the understanding of physiology and improved tools for genetic manipulation facilitated the systematic modification of metabolic pathways to produce dimeric and trimeric PHA directly from methanol without other additives. These products possess excellent mechanical properties, but this functional PHA yield still needs further improvement. Furthermore, the original strain could reach a high cell density (83.88 g_{CDW}/L), while engineered strains could only reach 30 g_{CDW}/L (Sonntag et al. 2015). The methanol concentration during fermentation or shake flask culturing was no more than 1% (v/v) (7.94 g/L), and tolerance to methanol for the MeCFs must be improved for the industrial application.

Metabolic pathways in AM1 grown on methanol are shown in Fig. 13.3. The carbon flux distribution has been investigated previously (Peyraud et al. 2009). About 22% of the carbon flux is used for acetyl-CoA synthesis, which is eventually used to form C2 biomass. The supply of acetyl-CoA is sufficient and stable. The EMC cycle consists of multiple branches that include saturation steps and consequently saturated compounds and chiral C4 and C5 acyl-CoA esters. AM1 has the potential to produce unique compounds from particular dicarboxylic acids such as (2S)-ethylmalonic acid, methyl fumarate and (2R/2S)-methylmalonic acid (Alber 2011). In 2014, Sonntag et al. (2014) discovered a bacterial thioesterase (YciA) that cleaves coenzyme A from the intermediate metabolite mesaconyl-CoA and (2S)-methylsuccinyl-CoA, which are specific to the EMC pathway in AM1. This work led to the successful production of methyl fumarate and 2-methylsuccinic acid from methanol. In 2014, Hu and Lidstrom (2014) successfully used crotonyl-CoA to produce 1-butanol by overexpressing crotonyl-CoA reductase and alcohol dehydrogenase, demonstrating the potential of AM1 in the production of biofuels. Interestingly, the engineered strain could only grow on ethylamine instead of methanol. In 2016, these researchers improved the 1-butanol tolerance up to 0.5% by laboratory evolution, and the 1-butanol titre was increased to 25.5 mg/L. In 2015, Sonntag et al. (2015) synthesized sesquiterpenoid α-humulene from methanol in a de novo manner by expressing α-humulene synthase from *Zingiber zerumbet* and farnesyl pyrophosphate (FPP) synthase from *Saccharomyces cerevisiae*, resulting in 18 mg/L α-humulene. By

Table 13.2 Chemicals produced by *M. extorquens*

	Product	Maximum concentration (yield)	Description	References
Amino acids	L-serine	54.9 g/L (8.3% from methanol, 39.3% from glycine)	Freeze-thawed resting cells with methanol and glycine as substrates	Sirirote et al. (1986)
		11.3 g/L (4.5% from methanol, 95% from glycine)	Immobilized resting cells with methanol and glycine as substrates	Sirirote et al. (1988)
PHAs	PHB (600 kDa)	8% (w/w)	Shake flask cultivation	Taidi et al. (1994)
	PHB (900−1.800 kDa)	52.9 g/L (0.09–0.12 g/g MeOH; up to 45% of cdw)	Methanol-limited (<0.1 g/L) fed-batch fermentation	Borque et al. (1995)
	PHB (size n.d)	149 g/L (0.2 g/g MeOH; 64% of cdw)	Fed-batch fermentation with defined carbon/nitrogen ratio	Suzuki et al. (1986)
		3.3 g/L (33% of cdw)	Fed-batch fermentation at 1.7 g/L methanol	Bourque et al. (1992)
	PHB-co-3HV	0.33 g/L (33% of cdw); ratio 0.62; 0.38	Shake flask cultivation with 0.5% methanol and 0.05% n-Amyl alcohol (v/v)	Ueda et al. (1992)
	PHB-co-3HV/ PHB-co-3HV-co-3HV	43% of cdw; ratio of 0.91; 0.06–0.03	Genomic substitution of native phaC by *Aeromonas caviae* phaC deletion of propionyl-CoA carboxylase (pcc); growth under cobalt limitation in shake flasks	Orita et al. (2014)
	Functionalized PHB	n.d.; up to 6% of C5:0, 6:5, 6:0, 8:7 or 8:0 monomers	Overexpression of native or heterologous phaC; C5–C11 saturated and unsaturated carboxylic acids as co-substrates	Höfer et al. (2010)
Dicarboxylic acids	Mesaconate	70 mg/L (0.0175 g/g MeOH)	Constitutive overexpression of acyl-CoA thioesterase YciA; shake flask cultivation	Sonntag et al. (2014)
	2-methylsuccinate	60 mg/L (0.015 g/g MeOH)		

(continued)

Table 13.2 (continued)

	Product	Maximum concentration (yield)	Description	References
Biofuel	1-butanol	15.2 mg/L	Ethylamine as sole carbon source; co-expression of adhE2 from *Clostridium acetobutylicum*, crotonyl-CoA reductase from *Treponema denticola* and endogenous croR	Hu and Lidstrom (2014)
		25.5 mg/L	Adaptive laboratory evolution was used to increase 1-butanol tolerance	Hu et al. (2016)
Terpenes	MEV	2.22 g/L	*pha*A gene from *R. eutropha* and *mva*S and *mva*E genes from *Enterococcus faecalis*. Further modification of the *phaA* expression by regulating the strength of the ribosomal binding site	Zhu et al. (2016)
	α-humulene	1.65 g/L	Expression of α-humulene synthase from *Zingiber zerumbet* in combination with farnesyl pyrophosphate (FPP) synthase from *Saccharomyces cerevisiae*, the prokaryotic mevalonate pathway from *Myxococcus xanthus*, using methanol-limited fed-batch cultivation	Sonntag et al. (2015)

introduction of a prokaryotic mevalonate pathway from *Myxococcus xanthus* in combination with the ribosome binding site optimization of α-humulene and FPP synthases, the α-humulene concentration was increased threefold and additionally raised by 30% using a carotenoid synthesis-deficient mutant strain to give final α-humulene concentrations of 1.65 g/L by methanol-limited fed-batch cultivation. In 2016, Zhu et al. (2016) introduced the mevalonate pathway into AM1 to achieve a high mevalonate production from methanol, which could offer a platform for terpenoid synthesis. Firstly, they constructed a natural operon (MVE) harbouring the *mvaS* and *mvaE* genes from *Enterococcus faecalis* and an artificial operon (MVH)

harbouring the *hmgcs1* gene from *Blattella germanica* and the *tchmgr* gene from *Trypanosoma cruzi*. This resulted in mevalonate concentrations of 56 and 66 mg/L by flask culture, respectively. Further introduction of the *phaA* gene from *Ralstonia eutropha* into the MVH operon increased the mevalonate titre to 180 mg/L, which was 3.2-fold higher than that achieved with the natural MVE operon. By regulating the strength of the ribosomal binding site, the mevalonate titre was increased to 215 mg/L, and fed-batch fermentation in a fermenter achieved a mevalonate titre of 2.22 g/L. With further study and the continuous improvement of tools for genetic manipulation, it is anticipated that multilevel gene regulation of AM1 could be achieved to establish an improved effective MeCF. Mevalonic acid is a key intermediate in the cells that offers a platform for the synthesis of many important compounds. It is also the precursor of more than 50,000 terpenoids via downstream pathway design. The production of mevalonate from methanol is thus the critical step that links methanol with valuable terpenoids by construction of MeCFs.

13.5 Prospects

Methanol can be diversely produced from biomass-originated biomethane, natural gas, shale gas and CO_2. Methanol as an alternative feedstock for bioeconomy can be competitive with sugar in terms of market price, and as a purely industrial product, methanol is not subject to strict use and price regulation or to import limitations imposed on the agricultural commodities such as corn or sugar (European Organization of the Markets in the Sugar Sector EG318/2006). Furthermore, reduced demand for the complex nutrients in methanol-based bioprocesses compared with that in traditional sugar-based bioprocesses results in lower costs for the culture media and also reduces the downstream processing cost, which can easily account for more than 50% of the manufacturing costs in conventional bioprocesses (Aldridge 2006; Xiu and Zeng 2008). Thus, processes for the production of value-added chemicals that start from methanol have been developed and look set to continue. The US Department of Energy (DOE) launched the Reducing Emissions using Methanotrophic Organisms for Transportation Energy (REMOTE) program in 2013 with a $34 million grant to promote the efficient use of methane and methanol for liquid fuel production, based on a promising outlook for methyl biosynthesis energy research (https://arpa-e.energy.gov/?q=arpa-e-programs/remote).

As shown in Fig. 13.4, industrial-scale bioprocess using methanol as a carbon source to produce single-cell protein (SCP) has proven successful, as demonstrated by Imperial Chemical Industries (ICI) which used *Methylophilus methylotrophus* to produce SCP with a maximum specific growth rate of 0.55 h^{-1}, a maximum cell concentration of 30 g/L and a cell yield of 0.5 g/g (Westlake 1986; Windass et al. 1980; Senior and Windass 1980). In this demonstration work, the methanol concentration was maintained at 0.026% (v/v) to avoid the methanol toxicity. Using a 1500 m^3 internal circulation airlift bioreactor, an annual production of 50,000 tons of SCP was achieved (Solomons 1985). In 1986, Hoechst and Uhde used *Methylomonas clara* to produce SCP, achieving an annual output of 1000 tons in a

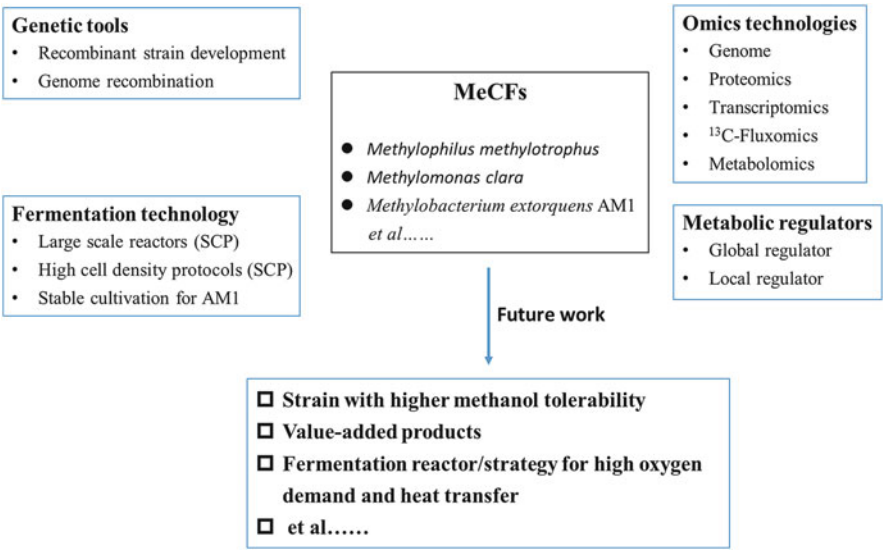

Fig. 13.4 Framework of key factors and future works of methanol-based industrial biotechnology

20 m^3 bioreactor, with a maximum specific growth rate of 0.5 h^{-1}, a maximum cell concentration of 5 g/L and a cell yield of 0.5 g/g and with a dilution rate of 0.3−0.5 h^{-1}. Again, the methanol concentration was maintained at 0.005% (v/v). In industrial-scale high-density cultivation processes, heat transfer and oxygen transfer are the limiting factors. In the Phillips/Provesta continuous production process, the fermentation broth is continually removed, and direct drying is employed in real time to solve both heat and oxygen transfer issues. As shown in Fig. 13.4, the omics analysis technology, genetic tools and metabolic regulators have been well established. Further technological advancements may further improve the performance of the bioreactors and fermentation processes using MeCFs.

Methanol toxicity to microorganisms reduces the risk of bacterial contamination in MeCF bioprocess but requires careful control of the substrate concentration at less than 1% (v/v), which always requires strict monitoring and constant feeding. The mechanism of MeCF tolerance to methanol is not well understood and is likely to be highly complex. A platform strain with a higher methanol tolerance could help to clarify the biological adaptation mechanism to methanol and would be potentially useful for industrial fermentation. Numerous studies have focused on the production of PHAs by AM1, and a fermentation strategy for controlling the ratio of carbon-nitrogen and cobalt ion deficiency has been developed based on the growth and metabolic characteristics of strains. A new design medium facilitates the stable growth of AM1 and improves the industrial-scale production of MEV (Cui et al. 2017). Industrial-scale fermentation using AM1 as the MeCF is therefore likely to be feasible, and other methylotrophic bacteria may also be needed to be explored for the industrial application in the future.

Acknowledgements This work was supported by the National Natural Science Foundation of China (NSFC 21376137) and the Tsinghua University Initiative Scientific Research Program (20131089238).

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Biogas, Bioreactors and Bacterial Methane Oxidation

14

Ilka Madeleine Mühlemeier, Robert Speight, and Peter James Strong

14.1 Methane Source: Fossil Methane Versus Microbial Methane

Pure methane is an energy-rich feedstock with an energy density of 55.7 MJ/kg (at 1.013 bar, 15 °C) and is used to generate electricity, for domestic heating and cooking or as a vehicle fuel. Although methane is the second most abundant greenhouse gas (GHG) after carbon dioxide, it has an impact at least 21 times greater. Approximately 18% of the total atmospheric radiative forcing is attributed to methane (EPA 2010; IPCC 2013). Methane is readily available for use as an energy source as it is the main component of natural gas and biogas. Natural gas is a fossil fuel that is abundant in oilfields and coal deposits, while biogas is widely produced during anaerobic microbial degradation of organic matter. Microbial synthesis of methane is of great environmental concern, as it constitutes the greater portion of the 60% of annual methane emissions attributed to anthropogenic activity (EPA 2010; Abbasi et al. 2012).

Methanotrophs, a subset of methylotrophs, are of increased scientific interest due to their ability to use methane as their sole carbon source. For biotechnological applications the source of methane is a contentious issue. Methane is readily available as a by-product from anaerobic digestion of organic material or reservoirs of natural gas. However, natural gas is negatively viewed from the aspect of environmental impact, as it is a fossil fuel that ultimately increases the total atmospheric radiative forcing. The term natural gas is an unfortunate misnomer, as it has been sequestered for such an extended time that it should be termed fossil methane.

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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_14

Nonetheless, it is a vast global methane reservoir. The World Bank estimates 92 Mt $year^{-1}$ of natural gas is wasted via flaring or venting (WorldBank 2015). This represents an enormous opportunity for converting methane into biological products. As the gas is released into the atmosphere regardless, it would be advantageous to convert the flared or vented gas into higher-value biological products or, at worst, ensure complete transformation into less harmful CO_2.

Methane derived from anaerobic biological activity (biogas) is viewed as a renewable resource and therefore the preferred source from an environmental perspective. Biogas composition and production volume may vary according to feedstock and the activity of a microbial consortium. The variability may be due to a heterogeneous substrate, inconsistent substrate and nitrogen supply, a significant change in pH beyond the optimal range of methanogens (±pH 6.8–7.5) or the build-up of inhibitory metabolites such as long-chain fatty acids or ammonia. Significant sources of renewable methane that are either captured or present the opportunity for capture and reuse include landfills (38 Mt/year), wastewater treatment (21 Mt/year), agriculture (11–30 Mt/year) and biomass (10 Mt/year) (Abbasi et al. 2012). Some of the problems associated with substrate variation and reactor performance can be overcome when there is a consistent residue supply and/or a co-substrate and an appropriately designed and monitored anaerobic digester and process.

The rapid expansion of global methane production and capture, both in the form of natural gas and biogas, has improved the accessibility of methane on the global market and consequently lowered the commodity price. Current government policy has also impacted the viability of biogas production. In Germany, for example, there are approximately 9000 biogas facilities which generate a gas containing 50%–60% methane. Until recently, these facilities were assisted by a governmental grant. Since the suspension of the grant in 2017 and implementation of new legislation regarding renewable energy, these plants are struggling to remain viable, and alternatives are being sought (EEG 2017). Elsewhere, such as the USA, access to vast reservoirs of natural gas by hydraulic fracturing has significantly lowered the commodity price of methane over the last decade (Kern and Characklis 2017). This is unfortunate, as the USA also has substantial crop residues associated with bioethanol production from corn (12.4 hm^3 $year^{-1}$), as well as capacity to grow biomass solely for biogas production (Kim and Dale 2016). The drop in price has strongly affected the broadscale adoption of anaerobic digestion as a technology to generate value from municipal organic wastes and agricultural residues. In Australia, even with large reservoirs of natural gas, anaerobic digestion is viewed with strong interest. This is because local electricity costs are high, and natural gas obtained by hydraulic fracturing is sold internationally (predominantly to China). However, changes in international demand, or political climate, can swiftly alter the economics of biological methane production. Internationally, biogas uptake has been inhibited by incentives towards carbon-mitigating technologies and limited views towards renewable energy, carbon pricing, environmental legislation and climate change. Uncertainty and volatility create wary investor sentiment, which can depress the development of biogas-generating facilities. Biogas facilities using methane to generate electricity or as a vehicle fuel are frequently marginal since the fall in commodity prices and removal of subsidies. A side-effect of this is an increased

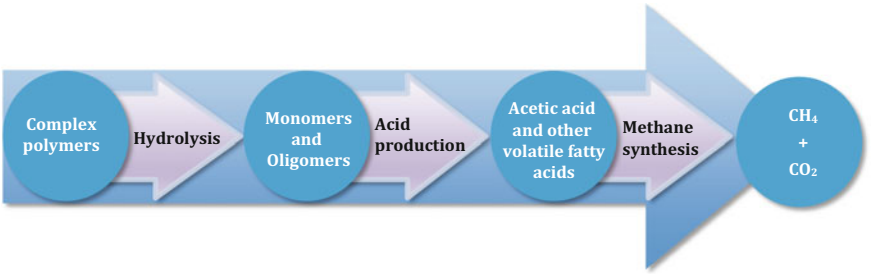

Fig. 14.1 Simplified illustration of methanogenesis from complex organic substrates

interest in novel, value-added processes that can transform methane. One such technology is the biological conversion of methane to higher-value products by methanotrophic organisms. This potential strategy for transforming biogas has recently gained significant traction. In the rest of this chapter, we will explore the production and use of methane derived from biogas and discuss the importance of reactor choice and its context to recent advances in industrial methanotrophy and environmental applications.

14.2 Sustainable Methane from Anaerobic Digestion

Methane produced during anaerobic digestion is viewed as a sustainable supply that is derived from a renewable resource. Microbial methane production and capture are an established technology for the treatment of organic waste streams in a number of industries such as municipal solid waste, wastewater treatment, brewing, food processing as well as agricultural and livestock residues (Abbasi et al. 2012). Anaerobic digestion is the anaerobic microbial catabolism of organic substrates that generates methane and CO_2 as the major metabolic by-products. The biological interactions during anaerobic digestion involve several types of microbes, each with their own requirements for optimal activity. Typically, anaerobic digestion proceeds according to the mechanisms illustrated in Fig. 14.1. An insoluble substrate (or complex soluble substrate) is enzymatically degraded and hydrolysed into simpler soluble compounds (hydrolysis) that are metabolised to generate organic acids (acidogenesis). These long- or short-chain fatty acids are converted into acetic acid (acetogenesis), which is transformed into methane by a consortium of bacteria and archaea (methanogenesis). Initially there is a higher conversion to CO_2 during acidogenesis, but as the microbial consortium reaches steady state, the biogas is composed predominantly of methane (up to 70%) and carbon dioxide (up to 50%). Hydrolysis of the complex material is recognised as the rate-limiting step in the digestion process (Appels et al. 2008). The biogas may contain other minor components such as ammonia, hydrogen sulphide or siloxane (Table 14.1).

Table 14.1 A comparison of methane from natural gas versus biogas from landfills or AD digesters

	Natural gas (fossil methane)	Biogas (anaerobic digestion)	
Advantages	+ Relatively consistent	+ Readily available/ widespread	
	+ Huge volumes available	+ Viewed as sustainable	
	+ Independent of microbial activity		
Disadvantages	− Fossil fuel	− Dependent on a microbial consortia	
	− Flared gas is frequently remote	− Varying consistency and volumes	
	− Less distributed		
Components	Natural gas	Landfill biogas	AD biogas
CH_4 (vol%)	81–89	30–65	53–70
CO_2 (vol%)	0.67–1	25–47	30–50
N_2 (vol%)	0.28–14	<1–17	2–6
O_2 (vol%)	0	<1–3	0–5
H_2 (vol%)	–	0–3	–
C_{1+} hydrocarbons	3.5–9.4	–	–
H_2S (ppm)	0–2.9	30–500	0–2000
NH_3 (ppm)	–	0–5	<100
Chlorines (mg N m^{-3})	–	0.3–225	<0.25
Siloxane (μg g^{-1})	–	<0.3–36	<0.08–0.5

Adapted from Yang et al. (2014)

The fraction of the waste that is degradable is important. Total solids is a parameter describing the mass of material within a liquid, which is composed of insoluble suspended solids and the dissolved material. Total solids (and solid waste residues) can be described in terms of volatile solids (the components that are volatilised at 550 °C) and ash (the incombustible component), which typically represent the organic and inorganic fractions of the waste, respectively. Ash content serves as a proxy to discern whether a residue is a potentially good or poor substrate for anaerobic digestion, as a high ash content is a disadvantage. The volatile solids component is not necessarily a good indicator of methane potential as it does not discern whether the organic fraction is composed of readily degradable compounds (e.g. simple sugars, proteins or fats) or recalcitrant compounds (e.g. complex biopolymers such as chitin or lignin). Substrates are typically assessed for their ultimate methane potential (UMP) using the BMP assay (biochemical methane potential, or biomethane potential assay). This assay gives two very important pieces of information: the greatest amount of methane that may be derived from a substrate under ideal conditions and the rate at which methane is produced. There are significant differences between the yields and the rates of methane synthesis from different organic substrates. As is evident in Table 14.2, the time taken to generate 80% of the UMP can vary from 3 days for milk to 23 days for a cattle waste slurry. The rate of

Table 14.2 Biochemical methane potential of different organic wastes and energy crops

	Total solids (TS) (g kg^{-1})	Volatile solids (VS) (g $kgTS^{-1}$)	Total CH_4 yield (L $kgTS^{-1}$)	Total CH_4 yield (L $kgVS^{-1}$)	UMP (L $kgVS^{-1}$)	80% UMP (L $kgVS^{-1}$)	Days for 80% UMP
Agricultural and livestock products or residues							
Grass silage	314	928	296	319	320	256	15
Maize silage	174	952	292	307	339	272	14
Mixed silage	294	920	272	296	307	247	13
Hay	913	937	268	286	292	233	19
Pig slurry	69.9	794	252	317	321	260	12
Cattle slurry	78	782	186	238	247	198	23
Food industry waste from dairy, distillery and cereal residues							
Cheese	562	960	633	659	659	530	8
Sour cream	265	992	708	714	717	570	4
Cottage cheese	265	980	590	602	602	481	6
Buttermilk	109	992	485	489	489	391	5
Milk (2.5% fat)	110	993	455	458	458	367	5
Milk (3.5% fat)	120	993	463	466	468	372	3
Raw milk	128	993	508	512	517	409	3
Distillery slop[a]	95.5	931	355	381	400	324	12

(continued)

Table 14.2 (continued)

	Total solids (TS) (g kg^{-1})	Volatile solids (VS) (g $kgTS^{-1}$)	Total CH_4 yield (L $kgTS^{-1}$)	Total CH_4 yield (L $kgVS^{-1}$)	UMP (L $kgVS^{-1}$)	80% UMP (L $kgVS^{-1}$)	Days for 80% UMP
Distillery slop[b]	55.4	912	306	335	385	310	17
Grain mill dust	874	940	256	272	274	235	13
Grain mill bran	794	914	300	328	330	281	11
Grain mill flour	912	896	344	384	386	315	10

Adapted from Luna-delRisco et al. (2011)
UMP ultimate methane potential
[a]Complete distillery slop
[b]Centrifuged distillery slop (i.e. no suspended solids)

production is a very important consideration as this influences the residence time and substrate loading of an anaerobic digester, ultimately impacting on reactor size. Additionally, it is not a simple case of loading a reactor according to a substrate's methane potential and rate of digestion. Although lipid-rich wastes have high ultimate methane potentials, reactors must be carefully maintained to circumvent inhibitory concentrations of long-chain fatty acids. Similarly with milk products, anaerobic digesters may be inhibited due to excessive ammonia production. There are various parameters that can inhibit the bacterial groups in anaerobic digesters, including pH, alkalinity, concentration of free ammonia, hydrogen, sodium, potassium, heavy metals and volatile fatty acids (Appels et al. 2008).

Landfills are viewed as a source of methane as they have historically received large quantities of organic material that degrades anaerobically when covered. The rate and yield of methane production are dependent on the amount of organic material present and its recalcitrance to biological degradation and are further affected by water availability and localised pH, temperature and oxygen ingress. Typically, only large landfills are capable of generating sufficient methane to justify the capital and operational expenditure required for methane capture and conversion into electrical energy. Landfill capping and gas collection infrastructure must be carefully designed to maximise methane extraction and minimise atmospheric loss and air ingress. Another consideration is the waste distribution and fracture zones in the site. This may result in methane seepage out of the capping material in localised areas—commonly referred to as hotspots (Ahoughalandari and Cabral 2016). Another consideration is the shift in public acceptance of landfilling—organic waste in particular. Currently, landfills may be a source of methane, but the long-term prospects are less promising. Organic waste that is separated at the source is increasingly popular and allows the residue to be diverted from landfills to processing facilities for composting or high-rate anaerobic digestion. This has environmental benefits, as less putrescible waste goes to landfills, and allows energy recovery from waste.

Large-scale anaerobic digesters treating diverted municipal waste, and other organic waste, represent the future of biogas synthesis. The solid residue remaining after anaerobic digestion can be used as a fertiliser/soil supplement. High-rate anaerobic digesters can control the substrate input and carefully maintain parameters that may inhibit the consortia involved in anaerobic digestion. Co-digestion, where various residues are blended, enables greater and more consistent methane production by tailoring the C-N-P ratio or dilution of inhibitory compounds. These digesters may utilise food waste, agricultural and livestock residues or even biomass specifically cultivated for biogas synthesis. They are also advantageous to landfill biogas in that they are relatively well sealed—and the positive pressure allows easier biogas removal. Landfills often use a vacuum to draw out methane, which allows air ingress into what is effectively a packed-bed bioreactor. This has significant implications for the bacteria involved: methanogens are obligate anaerobes that are adversely affected by oxygen. Anaerobic digesters can be designed to operate as single-stage or multistage systems that may use high or low suspended solid concentrations. They can operate in batch or continuous mode and operate at mesophilic or thermophilic temperatures. The configurations differ depending on

the main design focus of the plant, but a common configuration is the use of a two-stage system (decoupling acidogenesis and methanogenesis), operating under mesophilic conditions (Fendt et al. 2016). In addition, substrates can be treated prior to anaerobic digestion to enhance biogas production. Pretreatment methods include a range of possibilities, including thermal, mechanical, chemical (acid, base, oxidative), sonication and enzymatic hydrolysis (Appels et al. 2008).

14.3 Assimilation of Methane from Biogas

Using biogas to culture methane-oxidising bacteria has an advantage compared to its use as a fuel: contaminants do not inhibit microbial growth and therefore do not have to be removed. Contaminants in biogas used for heating, transport and electricity generation must be reduced to acceptable limits. Carbon dioxide and nitrogen content lower the calorific value and affect ignition behaviour, while sulfide can poison catalysts, corrode infrastructure, and transform into SO_2 after combustion. Ammonia has a detrimental effect on fuel cells and produces NO_x emissions after combustion. Siloxane forms silicon dioxide after combustion, which leads to mechanical corrosion in engines (Fendt et al. 2016). Methanotroph cultures can withstand high levels of contaminants. Zhang et al. (2016) isolated a methanotroph that could convert biogas to methanol without H_2S scrubbing. The isolate (potentially a *Methylocaldum* sp.) had a high H_2S tolerance and grew stably using methane/air mixtures that contained 500–1000 ppm H_2S. Methanol yields of 0.28–0.34 g L^{-1}, with methane-to-methanol conversion efficiencies of 30%–34%, were obtained using a biogas/air mixture containing 500 ppm H_2S. Even exposure to 1000 ppm H_2S did not affect methanol production.

Methanol synthesis and accumulation were evaluated in other studies using real or simulated biogas. Often yields were low, and electron donors (e.g. formate, H_2) and methanol dehydrogenase inhibitors (e.g. EDTA, $MgCl_2$, high salt concentrations and cyclopropane derivatives) were required to increase the yield (Yang et al. 2014). Recently, Sheets et al. (2016) isolated a methanotroph with characteristics comparable to obligate methanotrophs from the genus *Methylocaldum*. The isolate grew on biogas from a commercial anaerobic digester and synthesised methanol. Methanol production was enhanced by using several methanol dehydrogenase (MDH) inhibitors and formate as an electron donor. The maximum methanol concentration (0.43 g L^{-1}) and 48 h methane-to-methanol conversion efficiency (25.5%) were achieved using biogas as a substrate in a growth medium containing 50 mM phosphate and 80 mM formate. Yoo et al. (2015) assessed a simulated biogas (0.4% v v^{-1}) for methanol synthesis using *Methylosinus sporium* by inhibiting methanol dehydrogenase using phosphate, NaCl, NH_4Cl or EDTA. The maximum rate of methanol production was 200 μg mg^{-1} h^{-1} when the media contained 100 mM NaCl. Patel et al. (2016b) evaluated methanol production by *Methylosinus sporium* using raw biogas from an anaerobic digester that contained high concentrations of H_2S (0.13%). Methanol was produced using raw biogas, but the yield was low (0.16 g L^{-1}). Supplementing with H_2 and covalent immobilisation of the cells on chitosan improved methanol yield (0.23 g L^{-1}). Although these studies

highlight the potential for methanotrophic cultures to directly use biogas, they also highlight the low product yields from natural isolates—where the cost of the media alone may outweigh the value of methanol produced. Currently, it would take an enormous improvement in current process technology, as well as the isolate's synthesis capability, to obtain economically viable yields and production rates.

14.4 Gas Solubility and Mass Transfer

Even with an adequate supply of methane, factors such as mass transfer from the gas to liquid phase, reactor choice and operating conditions affect methane and oxygen availability, biomass yield and the rate of product synthesis. The greatest technical challenge for gas fermentation at scale remains an efficient transfer rate of poorly soluble gaseous substrates into the aqueous phase (Vega et al. 1990; Munasinghe and Khanal 2010a, b). The two primary substrates, methane and oxygen, have very poor solubility in water (Table 14.3). Gas solubility and the rate of transfer into an aqueous solution are influenced by a number of operating parameters, such as temperature, headspace pressure and media composition, gas superficial contact area and mixing. Gas solubility in an aqueous medium decreases as temperature increases. The solubility of oxygen in water at 5 °C is 1.8 mmol/L, while at 40 °C, the solubility drops to 1.03 mmol/L. Values illustrating these differences within the mesophilic range are displayed in Table 14.2. An increase in electrolytes also lowers gas solubility. For example, 2 mM NaCl in water at 25 °C lowers oxygen solubility by 40% (Chmiel 2011). Gas transfer into an aqueous medium is also affected by the difference in the partial pressures of the gas and the liquid phase. Bubble size also plays a significant role, as this affects the interfacial surface area. The gas mass transfer coefficient is described by the following equation:

$$\frac{dN_{GS}}{dt} = \frac{V_L \times K_L \times a}{H} \left(P_{GS} - P_{LS}\right) \tag{14.1}$$

where N_{GS} (mol) is transferred from the gas phase, V_L is the volume (L) of the reactor and P_{GS} and P_{LS} (atm) are the gas partial pressures in the gas and liquid phase, respectively. H (L atm mol^{-1}) is Henry's law constant, and a (m l) is the gas–liquid interfacial surface area for unit volume. The difference between the gaseous partial pressures in the gas and the liquid media is considered the main driving force for mass transfer and therefore controls the solubility of the substrate. Typically, the gas-liquid volumetric mass transfer is represented by the term $K_L a$—which represents the hydrodynamic conditions within a reactor and is a reliable parameter for comparing the effectiveness of gas transfer among different bioreactors (Munasinghe and Khanal 2010a, b).

An effective way to increase methane and oxygen mass transfer is to increase the reactor headspace pressure. Bubble size can be controlled to some extent by the sparger pore size and porosity. Bubble coalescence decreases the surface area and can be minimised to some extent using plate diffusers or reactor packing material

Table 14.3 Gas solubility for the primary gas substrates (O_2 and CH_4) and CO_2 by-product

Gas	Equation	Constants	Solubility (X_1)	Solubility (mg L^{-1})			
				5 °C	15 °C	25 °C	30 °C
Methane	$\ln X_1 = A + B/(T/100\ K) + C \ln (T/100\ K) \times DT$	$T/K = 303.15$ $A = -115.6477$ $B = 155.5756$ $C = -6.1698$	2.346×10^{-5}	0.034	0.026	0.021	0.019
Oxygen	$\ln X_1 = A + B/(T/100\ K) + C \ln (T/100\ K)$	$T/K = 303.15$ $A = -66.7354$ $B = 87.4755$ $C = 24.4526$	2.122×10^{-5}	0.061	0.048	0.039	0.0359
Carbon dioxide	$\ln X_1 = A + B/(T/100\ K) + C \ln (T/100\ K)$	$T/K = 303.15$ $A/B/C \Rightarrow$ derived from Henry's law constant equation	5.42×10^{-4}	2.77	1.97	1.45	1.25

Yaws and Braker (2001), Speight et al. (2005), and Serra et al. (2006)

that fragments the larger coalesced bubbles. The configuration of the headspace can have a significant impact on the reactor as the surface area allows for increased gas transfer (of substrates in, as well as by-products out). Operating at a higher pressure increases gas transfer as there is effectively a higher concentration in the gas phase, which increases the difference between the partial pressure of the gas and aqueous phases. For methane, adding paraffin or nanoparticles (described as methane vectors) to the medium can improve gas transfer. Inclusion of paraffin at 5% improved biomass accumulation of *Methylosinus trichosporium* OB3b sevenfold relative to a control without paraffin (Han et al. 2009).

14.5 Reactor Types and Configurations Used in Methane-Oxidising Cultures

Many reactor types have been used to study gas fermentation (e.g. continuous stirred-tank reactors, bubble column and airlift reactors, fluidised bed and trickle-bed reactors, packed-bed reactors and membrane bioreactors (Vega et al. 1990; Munasinghe and Khanal 2010a, b)), while improved gas transfer efficiency and/or operational safety has been assessed using microbubble generation (Munasinghe and Khanal 2010a, b) and immobilised hollow-fibre membranes (Hickey et al. 2011). The common reactor types are illustrated in Fig. 14.2, and their operation and application to methanotrophy are discussed further in this section and tabulated in Table 14.4.

14.5.1 Continuous Stirred-Tank Reactor (CSTRs)

CSTRs are the most extensively used bioreactors in laboratory research. They are well characterised, and their volumes commonly range from 1 to 5 L at a laboratory scale. They typically consist of a thick-walled glass vessel that is capped with a stainless steel lid, which contains ports for various probes, sampling lines, heating, gas supply and adding media components. Heating or cooling can be provided by an external jacket or via metal tubing that is in contact with the media. Agitation is provided by impellers, connected via a shaft to a stirrer that typically occurs above the lid. Mixing is improved by using baffles. Aeration is provided at the base of the reactor and can be as simple as a single tube or through a sintered sparger to provide finer bubbles.

Gilman et al. (2015) characterised *Methylomicrobium buryatense* 5GB1 in detail using a CSTR. They studied the effects of methane- and oxygen-limited conditions on growth and biomass content. The process was conducted at a mesophilic temperature (30 °C), using a high impeller speed (1000 rpm), under alkaliphilic conditions (pH 8.8). The methane source was premixed gas cylinders, which is typical of laboratory studies requiring precise inputs to characterise growth effects and calculate a mass balance. Gases can also be supplied separately, as this allows varying gas ratios to be studied with relative ease. Park et al. (1992) separately supplied air

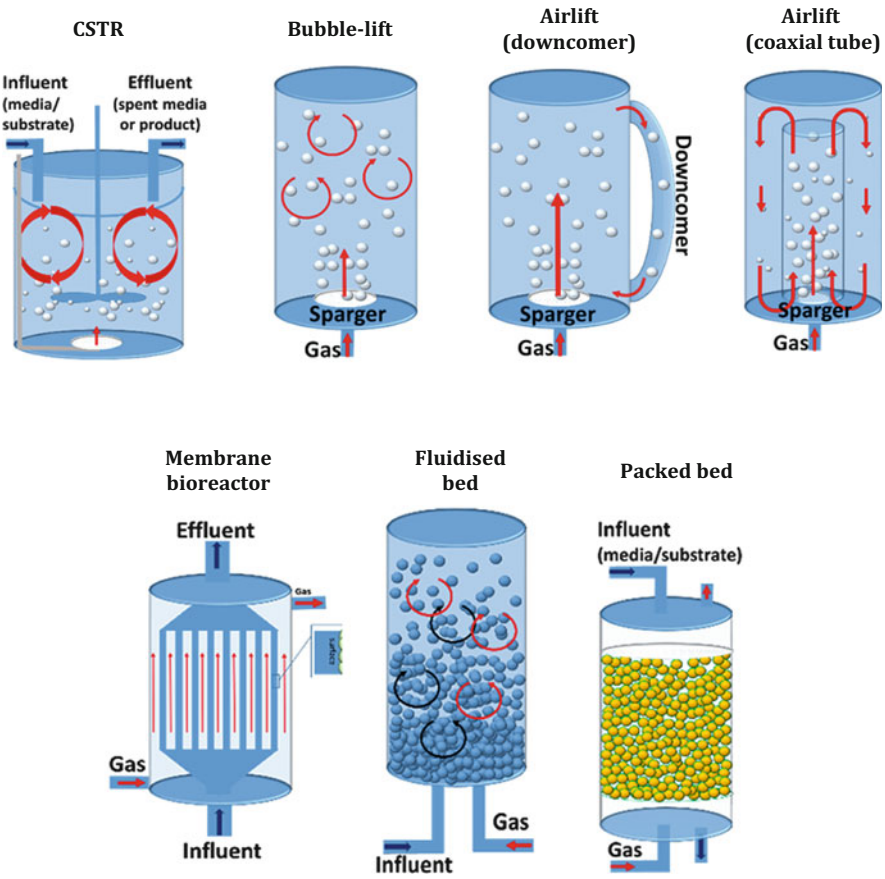

Fig. 14.2 Illustration of various bioreactor types, gas flow (red arrows) and influent/effluent flow (black arrows)

(600 mL min^{-1}) and methane (200 mL min^{-1}) to their reactor while optimising particulate methane monooxygenase (pMMO) synthesis by *Methylosinus trichosporium* OB3b. They used a CSTR at 30 °C, pH 6.8–7.2, an impeller speed of 500 rpm and a dilution rate of 0.06 h^{-1}. Park et al. (1991) had previously used a 5 L CSTR to optimise mesophilic soluble methane monooxygenase (sMMO) synthesis by *M. trichosporium* OB3b (30 °C; pH 6.7–7.8). Takeguchi et al. (1999) studied the effect of iron and copper on pMMO synthesis by *M. trichosporium* OB3b using a 5 L CSTR with a liquid volume of 2.3 L, at 30 °C, with the headspace containing 50% methane and 50% oxygen.

Table 14.4 Reactor descriptions, advantages, disadvantages and relevant literature

Reactor	Short description	Advantage/ disadvantage	Organism and product/ process
Bubble-lift and gas-lift bioreactors	A column reactor where the medium is agitated by a gas pneumatically compressed through a perforated sparger at the reactor base. Bubble-lift reactor mixing is random and caused by ascending bubbles, while airlift reactors rely on a change in density to enable mixing—usually with a dispersion, a tube is installed coaxially in the reactor or a sidearm	+ Good liquid-gas ratio + Good mass transfer + Simple construction − Pressure loss caused by the hydrostatic height − High shear effects − Potentially explosive gas mixture in the headspace	Literature available for synthesis gas fermentation using organisms other than methanotrophic bacteria − Bredwell et al. (1999) and Munasinghe and Khanal (2010a, b)
Continuous stirred-tank bioreactor (CSTR)	Reactor with a mechanical stirrer. The shaft is normally rotated using a motor above the reactor. Impellers provide the mixing. Gas is normally sparged at the base of the reactor	+ Well studied over a long period of time + Easy to use in small scale	− Organism: *Methylomicrobium buryatense* 5GB1 − Achieving precise bioreactor-based datasets (Gilman et al. 2015)
		− Less effective mixing at large scale − High mechanical shearing stress − High energy input − Foaming − Potentially explosive gas mixture in the headspace	− Organism: *Methylosinus trichosporium* OB3b − Production of pMMO (Park et al. 1992; Shah et al. 1996; Takeguchi et al. 1998, 1999) − Production of sMMO (Park et al. 1991)
Membrane-based bioreactors	Stirred-tank reactor that included a membrane for bubble-free aeration	+ Good substrate supply + Low risk of building an explosive gas mixture	− Organism: *Methylosinus trichosporium* OB3b − Production of methanol − Ref. Pen et al. (2014)
		− Poor scalability − Membrane fouling	− Organism: *Methylosinus trichosporium* OB3b − Production of methanol (Duan et al. 2011)
	Stirred-tank reactor combined with a hollow-fibre membrane for control	+ Easy to use at lab scale + Cell retention	− Organism: *M. capsulatus* (Bath) − Reason: production of pMMO (Yu et al. 2003)

(continued)

Table 14.4 (continued)

Reactor	Short description	Advantage/ disadvantage	Organism and product/ process
	of the media hydraulic retention time	via the hollow-fibre membrane	
		− Inhomogeneity at large scale − High mechanical shearing stress − High energy input − Foaming − Potentially explosive gas mixture in the headspace	
	Reactor with immobilised organisms or enzymes that are adsorbed, covalently bound or cross-linked to the membrane surface	+ Gentle culture conditions for the organisms or enzymes + Consistent substrate supply + Easy product removal	− Organism: *Methylosinus trichosporium* OB3b − Production of methanol (Patel et al. 2016a)
		− Cost intensive − Only for high value-added products	− Enzyme pMMO from *M. capsulatus* (Bath) − Reason: production of methanol (Blanchette et al. 2016)
Fluidised bed bioreactor	Reactor containing cell granules or cells immobilised on carriers having a density greater than the culture medium. Dispersion/mixing is maintained by recirculating media or gas sparging	+ No washout of cells during continuous fermentation	− Organism: *Methylocystis* sp. − Reason: trichloroethylene degradation (Shimomura et al. 1997)
		− Only for synthesis of extracellular products − Additional costs for immobilisation	
Trickle-filter/ trickle-bed bioreactor	Reactor system for wastewater treatment. The rector system contains a fixed bed reactor. For aeration, gases are pressed from the bottom through the fixed bed. For wastewater treatment and to provide the fixed bed for clogging, the wastewater flows down the fixed bed	+ Simple and reliable process + Low cost + Low energy	− Organism: *Methylosinus trichosporium* Ob3B − Reason: atmospheric methane removal (Yoon et al. 2009)
		− Clogging of interstitial pores	− Organism: *M. sporium* − Reason: methane mitigation (Lebrero et al. 2015)

14.5.2 Bubble-Lift and Gas-Lift Bioreactors

Gas-lift/bubble-lift reactors supply compressed gas at the base of the reactor to the liquid. Here, aeration by ascending bubbles causes mixing in bubble-lift reactors. With airlift reactors, a coaxial tube in the reactor, or an external side arm, enables homogenous mixing by a density differential. This extends the retention time of the bubbles in the reactor, which improves gas utilisation. Modifications of the traditional bubble- and gas-lift reactors are used in large-scale methane and synthesis gas (syngas) fermentation processes. The mass transfer of gases in these reactors, coupled to significantly lower energy inputs, make them more appealing than CSTRs at larger scales. The achievable range of $k_L a$ using microbubble spargers is 200–1800 h^{-1}, which is much higher than the range of CSTRs of 10–500 h^{-1} (Bredwell et al. 1999). At larger scale, the reactors rely on a pump to force liquid flow along the downcomer. Gas can be introduced against this flow direction to increase the pressure and the gas holdup time to improve mass transfer and utilisation. Operating at an overpressure in the headspace can further enhance gas mass transfer. A U-loop reactor was patented for the production of BioProtein A/S that consisted of pipes arranged in a U-shape, with a large headspace unit connecting the lower U-tube. The design for a 10 m^3 reactor could operate at a pressure of up to 5 bar. A dilution rate of 0.2 h^{-1} was used for continuous growth, with a reported oxygen concentration maintained between 10 and 25 ppm while operating at 45 °C and pH 6.5 (Larsen 2002). Alternatively, the reactor can also be placed horizontally, as opposed to the vertical U-loop reactor.

14.5.3 Membrane Bioreactors

In the more classical sense, MBRs were used to prevent cell washout from a bioreactor, as they improved retention of active biomass and made it easier to manipulate the media retention time. MBRs can contain cells freely suspended on solution or immobilised as a biofilm. They are frequently used in wastewater applications such as high-rate anaerobic digesters, where a high cell density of slow-growing microbes is maintained for carbon and nutrient removal. Mehta et al. (1991) used a 50 mL ultrafiltration cell as a CSTR that contained *Methylosinus trichosporium* OB3b cells bound to the DEAE-cellulose membrane to produce methanol. Methane and oxygen were provided at a ratio of 1:1, and the experiments were conducted at 35 °C, pH 6.4, in a 100 mM phosphate buffer containing 5 mM $MgCl_2$. Yu et al. (2003) coupled a hollow-fibre membrane reactor with a CSTR to culture *Methylococcus capsulatus* (Bath). They were able to closely control the copper ion concentration and biomass content using a 5 L reactor at a controlled pH (6.8–7.4). Impeller speeds ranged from 200 to 800 rpm, and the methane and airflows ranged from 0.7 to 1.3 L min^{-1}.

The gases used in methane oxidation are a health and safety concern because methane in air has a 5% lower explosive limit and an upper explosive limit of 15% (Yaws and Braker 2001). Membrane bioreactors have a distinct advantage to many

reactor configurations as they allow bubble-free gas transfer—thereby not generating explosive gas bubbles. This is not a membrane bioreactor in the classical sense, so much as a CSTR using membranes to enable gas-liquid contact. In this manner oxygen and methane can be supplied into the liquid separately; hence a premixture of the gases is unnecessary, and explosive mixtures are avoided. Pen et al. (2014) used membrane-facilitated gas transfer for the production of methanol by *Methylosinus trichosporium* OB3b. They used a very small stirred-tank reactor (100 mL) combined with two gas/liquid membrane contactors operating at 25 °C at pH 7.0. They obtained a twofold increase in mass transfer compared to a normal batch reactor and were able to avoid gas bubbles and dangerous gas mixtures during operation. Duan et al. (2011) also reported the production of methanol by *Methylosinus trichosporium* OB3b in a membrane bioreactor where two dense silicon tubes were used for gas transfer. They varied the methane-air ratio from 10:1 to 5:1, and impeller speed varied as required while operating at 30 °C, pH 7.0. They obtained a relatively high concentration of methanol (1.1 g L^{-1}) under optimised conditions (17 g L^{-1} dry mass, 400 mM phosphate and 10 mM Mg^{2+}) in the presence of 20 mM formate. However, a large surface area is required, and this can be prohibitively expensive at large scale. Also, the transfer of gas is not only a function of the membrane pore surface area but the diffusion of gas from the membrane lumen across the length of the pore. If a liquid boundary layer is established on the outer membrane, it can saturate with gas quickly, decreasing the gas concentration differential, and result in poor mass transfer (Munasinghe and Khanal 2010a, b).

14.5.4 Fluidised Bed Reactor

Another method to maintain cell density in a bioreactor is to immobilise cells on carriers or to each other as granules. The carriers, or cell granules, are kept in suspension by recirculating fluid or gas at the base of the reactor and are known as fluidised bed reactors. Shimomura et al. (1997) immobilised methane-oxidising bacteria in alginate gel beads (1.5 g dry weight on each bead) that were used in a 20 L fluidised bed bioreactor. A stainless steel mesh prevented bead washout. The media flow rate was 130 L min^{-1}, and a premixed gas mixture was used to feed the bacteria. The gas was supplied at the base of the reactor at a flow rate of 100 mL min^{-1}, while headspace gas was recirculated at a flow rate of 3 L min^{-1}, to fluidise the gel beads within the medium. Immobilised cells are not only easy to recover and recycle but can be significantly more stable than their free suspension counterparts. Immobilisation or encapsulation is a promising approach to improve the stability of methanol production. Patel et al. (2016a) immobilised methanotrophs in alginate and silica gels for methanol synthesis (pH 7.0, at 30 °C). Yields were low (approximately 0.11 g L^{-1}), but the immobilised cells retained significantly greater activity after six cycles (52%–62%) than the suspended cells (11%).

14.5.5 Trickle-Filter and Packed-Bed Bioreactors

Another design that makes use of immobilised cells is a trickle-filter bioreactor. Here, cells are immobilised on a surface (inert or non-inert), and a thin layer of media flows across the biofilm surface. This type of a reactor has very low energy requirements (especially if gravity-fed), and the thin film allows for rapid diffusion of gas from the liquid to the cells. Lebrero et al. (2015) investigated a two-phase trickle filter for methane mitigation. They used a column reactor (4 L working volume, 100 cm high, 80 cm in diameter) packed with 1 cm^3 polyurethane foam (PUF) cubes. Methane-laden air was supplied at the column base (1 L min^{-1}), and the addition of a nonaqueous phase enhanced methane removal. Essentially, a trickle-bed reactor is similar to a fluidised bed reactor, except that the carrier or support that the cells are immobilised on becomes the internal support structure because of the lack of liquid media. This design can be taken a step further, where no liquid media are actively passed over the immobilised cells and support structure, known as a packed-bed reactor. This is commonly used for passive bioremediation systems and is also known as a biofilters. Biofilters can be used in soil matrices or as floating structures above lagoons for mitigating atmospheric methane emissions and are discussed in Sect. 14.6.

14.5.6 Immobilised Enzyme Reactor

Instead of whole-cell cultures, isolated, or crudely purified, enzymes could be immobilised and used for methane oxidation. Blanchette et al. (2016) immobilised free pMMO from *Methylococcus capsulatus* (Bath) in a PEG hydrogel and assessed temperatures between 25 °C and 45 °C, at gas and liquid flowrates of 0.5 and 0.75 mL h^{-1}. During these experiments, enzyme activity was retained in the polymer construct (Blanchette et al. 2016). To some extent, this is a step towards the holy grail of methane oxidation, being able to transform methane using only the enzyme (or the catalytic centre) under benign environmental conditions. However, there are still significant advances required to achieve this, including extended enzyme activity, the ability to perform the oxidation without having to replenish the reducing agent (NADH) and providing a cost-effective high surface area reactor. While microfluidic chambers allow for conceptual validation, enzyme catalysis could conceivably be performed in variants of any number of reactors, including membrane, trickle-bed or even fluidised bed reactors (Table 14.4).

14.6 Greenhouse Gas Mitigation Versus Product Synthesis

From an applied perspective, the oxidation of methane may be viewed as two extremes: a biotechnological application for the synthesis of products or environmental bioengineering for mitigating atmospheric methane emissions from anthropogenic activities and, to a lesser extent, bioremediation of environmental

contaminants. Methanotrophs are capable of transforming nitrogen in wastewater treatment systems and oxidising various hydrocarbons, including aromatic compounds and halogenated aliphatics because of the broad substrate range of their methane-oxidising enzymes. There has been progress in the use of methanotrophs to synthesise products such as single-cell protein and biopolymers and, encouragingly, while using biogas as the source of methane.

14.6.1 Mitigation and Bioremediation

Agriculture (including livestock farming), waste management (including landfilling) and fossil fuel retrieval, processing and delivery (including coal mining) are the three largest sources of anthropogenic methane emissions (Hanson and Hanson 1996). Biological methane oxidation is vitally important as methanotrophs oxidise up to 40 Tg CH_4 yr^{-1} and sequester more than 50% of the methane produced in soils (Reeburgh et al. 1993; IPCC 2013). The ability of the methanotrophs to lower methane emissions and degrade hazardous organic compounds is greatly affected by factors such as methane and oxygen concentrations, temperature, nutrients and moisture availability (Jiang et al. 2010). In agricultural sediments and soils, naturally occurring methanotrophs sequester methane, but they are affected by fertiliser applications. Here, an interesting alternative is to prevent methane synthesis rather than degrade it. The "prevention is better than cure" approach is best for many environmental problems. With rice production, adding biofertilisers, or changing crop practices, can inhibit methanogenic synthesis of methane (Singh and Strong 2015). Similarly, the beef and dairy industry is a major methane emitter, and, while there has been little advancement with regard to applying methane-oxidising bacteria, the addition of a seaweed supplement to their feed has demonstrated very promising results in significantly lowering methane emission (Kinley et al. 2016). In an interesting demonstration of residue recycling, Wu et al. (2017) evaluated the conversion of digester spent solids into activated carbon, which was subsequently used to immobilise methane-oxidising bacteria in a biofilter that scrubbed methane from the vented gas from a biogas upgrading process. The immobilised methane-oxidising bacteria successfully eliminated methane from exhaust gas from a biogas upgrading process, at a methane removal efficiency of approximately 400 g h^{-1} m^{-3}, which was more than fourfold higher than that of free cells.

Methanotrophic methane mitigation technologies have been demonstrated beyond the laboratory as adaptable field-scale systems that can be engineered to meet site-specific variations and consistently minimise atmospheric methane emission. Engineered biosystems that use naturally occurring methanotrophs for methane mitigation include biocovers, passively or actively vented biofilters, biowindows and biotarps (Huber-Humer et al. 2008; Scheutz et al. 2009). These typically consist of a compost layer that allows oxidation to occur above an engineered gas distribution layer (Scheutz et al. 2009). The gas distribution layer is typically composed of a coarse material such as gravel, which enables homogenous gas permeability. The efficiency of methane oxidation in these passive methane oxidation biosystems is

strongly governed by the intensity and distribution of CH_4 at the base of the methane oxidation layer, which is strongly affected by the saturated hydraulic conductivity and pore size distribution of the material, and these effects are intensified as the slope of the interface increases. A well-designed passive methane oxidation biosystem has unrestricted upward flow of biogas across the interface between the gas distribution layer and the methane-oxidising layer, and moisture is uniformly distributed—thereby preventing methane overloading and hotspots of methane emission (Ahoughalandari and Cabral 2016). The advantages of passive methane oxidation technologies include low capital and operational expenditure, low maintenance requirements and effective use of organic waste materials like yard waste, biowaste or sewage sludge for biocover construction (Scheutz et al. 2009). The disadvantages include the dependency on environmental factors influencing CH_4 oxidation resulting in fluctuating CH_4 removal efficiencies (Scheutz et al. 2009), the lack of composting and decrease in CH_4 removal efficiency at colder temperatures (Lou and Nair 2009), an inability to efficiently mitigate large flow rates that may occur in hotspots (Manfredi et al. 2009) and the inability to recover energy. Nonetheless, biological mitigation technologies are adaptable systems that may be engineered to meet site-specific climatic variations, thereby ensuring minimal atmospheric emissions (Huber-Humer et al. 2008) as 100% oxidation efficiencies have been reported for field-scale applications (Nikiema et al. 2007; Gebert et al. 2009). In addition to methane removal, consortia species can degrade malodourous volatile organic compounds, such as trimethylamine and dimethyl sulphide (Lee et al. 2017) and toluene (Su et al. 2015).

14.6.2 Product Synthesis

Methane-oxidising bacteria synthesise a wide variety of compounds, which include biopolymers (PHB and glycogen), surface layers (external protein structures), soluble metabolites (methanol, formaldehyde, organic acids), osmolytes (sucrose and ectoine), lipids (biofuel and dietary supplement), enzymes (dehydrogenases, oxidase and catalase) with high catalytic efficiencies, copper-binding proteins (methanobactin: Dassama et al. (2017)) and pharmaceutical and antimicrobial proteins, while genetically engineered methanotrophs can synthesise carotenoids, isoprene or farnesene (Strong et al. 2015, 2016). In addition, their methane-oxidising enzymes have a broad substrate range and can co-oxidise compounds such as propylene to propylene oxide. Genetically modified methanotrophs are generally viewed as the most likely candidates for commercial production of chemicals. Lactic acid and isobutanol are two bioproducts that have enjoyed significant media coverage. Lactic acid (Calysta) is a monomeric precursor to for poly-lactic acid, while isobutanol (Intrexon) is a fuel additive.

To date, single-cell protein has appeared the most viable product from methanotrophs (references in Strong et al. (2015)), but media coverage (although inherently biased) indicates that the scale barrier and commercial production are potentially surmountable. A US biotechnology startup, Calysta, has secured

multimillion dollar funding between 2015 and 2017 in both the USA and UK to commercialise single-cell protein production. Calysta acquired the rights to BioProtein A/S, originally a product developed by Norferm, a Norwegian subsidiary of Statoil (with DuPont having a 50% stake). Norferm's commercial reactor produced thousands of tons of protein per year that was sold as a supplement to fishmeal, but production ceased in 2006. A joint venture between Cargill Inc. and Calysta Inc. plans to build a commercial facility in Memphis, Tennessee, which could be the world's largest gas fermentation facility. As of May 2017, Calysta had shipped 4 tons of product from their pilot facility at Teesside in the UK. Also advancing gas fermentation on a larger scale is Intrexon Corp. Its pilot plant in southern San Francisco, dedicated to the production of isobutanol, was operational in early 2016. Biopolymer synthesis using biogas has been explored with greater intent over the past decade. Mango Materials, also based in San Francisco, aims to commercialise polyhydroxybutyrate (PHB) production from biogas. Newlight Technologies, also in California, has claimed advanced, cost-efficient production capabilities of biopolymer-based thermoplastics using biogas as a substrate and a biological catalyst. However, information is minimal with regard to their catalytic process, which may be microbial or enzymatic. Newlight recently announced a large volume deal with IKEA, where Newlight's commercial-scale production facilities will enable IKEA to produce its trademarked AirCarbon thermoplastic under a technology licence, first using biogas and then potentially carbon dioxide. This will be a significant advancement for methane transformation if Newlight can produce a biopolymer from biogas, at commercial-scale and at a threshold that is competitive with petroleum-derived plastics.

Potential obstacles for an economically viable process may include suitable production strains, reactor size and costs, provision of the co-substrate (oxygen), gas storage and the lower explosive limit of methane, besides downstream processing costs associated with separation, concentration, purification and processing. How these can be overcome will be scrutinised in the near future. The demonstration of commercial-scale synthesis of single-cell protein, biopolymers (or their precursors) or even isobutanol, which is economically viable and independent of fossil fuel prices and government legislation, will be a major technological leap forward for applied methanotrophy.

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15

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Abbreviations

1,1-DCE	1,1-dichloroethylene
AME-D	aerobic methane oxidation coupled to denitrification
BTZ	benzotriazole
cDCE	*cis*-l,2-dichloroethylene
CSTR	continuous stirred tank reactor
DMSO	dimethyl sulfoxide
DO	dissolved oxygen
DW	dry weight
HCFCs	hydrochlorofluorocarbons
HFCs	hydrofluorocarbons
LAS	alkylbenzene sulfonate
MMC	mixed methanotrophic consortium
MMCs	mixed methanotrophic consortia
MMO	methane monooxygenase
MOB	methane-oxidizing bacteria
MOC	methane oxidation capacity
NMS	nitrate mineral salt
PFR	plug flow reactor
PHA	polyhydroxyalkanoate
PHB	polyhydroxybutyrate

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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_15

pMMO	particulate methane monooxygenase
RuMP	ribulose monophosphate
SCP	single cell protein
sMMO	soluble methane monooxygenase
SMX	sulfamethoxazole
TCE	trichloroethylene
t-DCE	*trans*-1,2-dichloroethylene
TT	trehalose and tryptic soy broth
VC	vinyl chloride
VSS	volatile suspended solids

15.1 Introduction

The widespread aerobic methanotrophs, or methane-oxidizing bacteria (MOB), can use methane as the sole carbon and energy source. As the only biological methane sink in the nature, methanotrophs oxidize approximately 10% of the annual methane emission and play an important role in the global carbon cycle (Hanson and Hanson 1996). The oxidation of methane to methanol is catalyzed by methane monooxygenase (MMO), which contains a soluble cytoplasmic form (sMMO) and a particulate membrane-associated form (pMMO). Besides methane, MMO can also use alkanes, alkenes, and many other compounds as substrates for oxidation reactions (Jiang et al. 2010).

Till now, the known aerobic methanotrophs are classified into 21 genera (Nazaries et al. 2013). According to the morphological, physiological, biochemical, and other characteristics, methanotrophs have traditionally been grouped into two "types," i.e., Type I and II (Semrau et al. 2010). Generally, Type I methanotrophs are *Gammaproteobacteria* that use the ribulose monophosphate (RuMP) pathway for formaldehyde assimilation, while Type II are *Alphaproteobacteria* that use the serine pathway (Strong et al. 2015). Most methanotrophs prefer to grow at moderate pH (6–8) and temperature ranges (20–40 °C), but extremophilic methanotrophs including thermophiles, psychrophiles, alkaliphiles, acidophiles, and halophiles were also discovered (Nazaries et al. 2013; Trotsenko and Khmelenina 2002). In addition, facultative methanotrophs in the genera *Methylocella*, *Methylocapsa*, and *Methylocystis* have been found to grow solely on C_2 to C_4 organic acids or ethanol (Semrau et al. 2011). These discoveries expand our knowledge of methanotrophy and indicate that its diversity as well as metabolic pathway flexibility might be greater than previously thought.

Methanotrophs have promising applications in the fields of environmental bioengineering and biocatalysis. Strong et al. (2015) comprehensively summarized the potential products (single cell protein (SCP), biopolymers, soluble metabolites, lipids, etc.) and processes (bioremediation, chemical transformation, wastewater

denitrification, electricity generation, etc.) that could be driven by methane-oxidizing bacteria. There are also some specific reviews referred to the related subjects including production of biopolymer (Karthikeyan et al. 2015; Khosravi-Darani et al. 2013; Strong et al. 2016), bioremediation of heavy metals and organic pollutants (Pandey et al. 2014), mitigation of methane emission (Jiang et al. 2010), degradation of chloroform (Cappelletti et al. 2012), and metabolism (Kalyuzhnaya et al. 2015; Trotsenko and Murrell 2008).

However, the limited available methanotrophic strains, slow growth rate, low cell density, complex MMO purification process, and poor enzyme stability make the applications of methanotrophs and MMO difficult (Jiang et al. 2016a). In addition to pure culture of methanotrophs, employing a mixed methanotrophic consortium (MMC) is another approach for MOB applications. A MMC generally is comprised of predominant methanotrophs and other companion microorganisms. The coexisting bacteria are supposed to remove toxic metabolites (e.g., formaldehyde and methanol) or supply key nutrients for methanotrophs (Helm et al. 2006). Methylotrophs, which utilize not only methane but also other one-carbon substrates (methanol, methylamine, formate, and formaldehyde), are the most common bacteria companying methanotrophs. Usually, the mixed methanotrophic consortia (MMCs) have better growth, higher cell density and methane-oxidizing activity, stronger stability, and environmental adaptation and could be applied in open system. This chapter will focus on research progress of the applications using MMCs, especially in the fields of environmental bioengineering and biocatalysis. The contents of MMCs' acquisition, interactions among methanotrophs and the coexisting heterotrophs, and highlight researches will be covered and discussed.

15.2 Acquisition of MMCs

For basic and practical application, obtaining MMCs with good activity and stability is the first issue. Methanotrophs can be widely found from muds, swamps, rivers, rice paddies, oceans, soils, woods, sewage sludge, etc., especially in the methane-rich environments (Hanson and Hanson 1996; Pandey et al. 2014). MMCs can be easily acquired by enrichment culture of these samples (Jiang et al. 2016b; Shukla et al. 2010; van der Ha et al. 2010) or mixing different isolated methanotrophs together (Pieja et al. 2012; Zhang et al. 2009). Table 15.1 summarizes recent researches about MMCs obtained by these methods.

In our previous studies, artificial coal bed gas (ACG, composed of CH_4, CO_2, C_2H_6, and C_3H_8) and methane were applied to enrich MMCs from the soil of a coal mine, respectively (Jiang et al. 2016b). The methane oxidation capacity of the cultures appeared stable after 6 subcultures, while the bacterial communities reached stability more slowly, around 24 subcultures. The multi-carbon sources in ACG resulted in more varieties of bacteria, but did not help to maintain the diversity or to increase the quantity and activity of methanotrophs. Therefore, methane is the ideal carbon source for enrichment with the purpose of maximizing methanotrophs and methylotrophs. A scale-up fermentation of the MMC enriched by methane was

Table 15.1 Summary of recent researches about MMCs obtained via enrichment and mixing

Community of MMC	Acquisition	Condition	Biomass, activity, and function	Reference
Dominant genus: *Methylocystis*	Enriched from soil of agriculture farm	NMS medium, 37 °C, 0.2% (v/v) methanol, inoculated in biofilter for 30-day acclimation	Max. TCE removal efficiency over 90%, max. TCE elimination capacity 6.7 g/m^3 h	Shukla et al. (2010)
MMC contained Type I and Type II	Enriched from landfill soil	NMS medium, 25 °C, CH_4: CO_2: air ~ 3:1:6	OD_{600} 0.32, max. MOC 495 mg CH_4/g $DW_{biomass}$h, 4.8% PHB	Chidambarampadmavathy et al. (2015)
MMC contained Type I and Type II	Enriched from compost soil	NMS medium, 25 °C, CH_4: CO_2: air ~ 3:1:6	OD_{600} 0.41, max. MOC 480 mg CH_4/g $DW_{biomass}$h, 2.1% PHB	Chidambarampadmavathy et al. (2015)
Type I:Type II ~ 1:1 Dominant genera: *Methylomonas*, *Methylocystis*, *Methylophilus*	Enriched from coal mine soil	NMS medium, 5 μM Cu^{2+}, 30 °C, CH_4: air ~ 1:5	2.69 g $DW_{biomass}$/L (OD_{660} 3.9), max. MOC34 mg CH_4/g $DW_{biomass}$h (still)	Jiang et al. (2016a, 2016b)
MMC	Enriched from marine sediment	NMS medium, 25 °C,CH_4: air ~ 1: (3–4)	0.6 g $DW_{biomass}$/L, CH_4 removal efficiency 80–99% at gas of 0.1 L/min with 1% CH_4 in biofilter	Karthikeyan et al. (2016)

(continued)

Table 15.1 (continued)

Community of MMC	Acquisition	Condition	Biomass, activity, and function	Reference
M. trichosporium OB3b, *M. trichosporium* IMV3011, *M. capsulatus* HD6T, and *Methylomonas* sp. GYJ3	Mixture	NMS medium, 30 °C, CH_4: air ~ 1:1	No advantages in activity of propene epoxidation, activity of naphthalene oxidation, and ability in synthesis of PHB comparing to isolated MOB	Zhang et al. (2009)
M. parvus OBBP, *M. trichosporium* OB3b, *M. hirsuta* SV97, *Methylocystis* 42/22, and enriched culture WWHS	Mixture	NMS medium, 1 μM Cu^{2+}, 30 °C	OD_{670} 3.9, 20–25% PHB, nitrogen and methane limitation increased PHB production, dominated by *M. parvus* OBBP with no detectable minority populations	Pieja et al. (2012)

NMS nitrate mineral salt (Whittenbury et al. 1970), *DW* dry weight, *MOC* methane oxidation capacity, *PHB* polyhydroxybutyrate

carried out from 5 L to 100 L and then to 600 L fermenter, and the biomass of 2.69 g dry weight (DW)/L was obtained in the 600 L fermenter after 25 h cultivation, indicating the feasibility for large-scale and rapid preparation of MMCs (Jiang et al. 2016a). The coexisting heterotrophs played an active role in promoting bacterial growth and MMO activity of the mixed culture. In each growth cycle of this methane-driven MMC, a regular process that methanotrophs use methane to grow preferentially, and then the other methyl bacteria as well as non-methyl bacteria will grow. This cyclical shift makes the community and function of the mixed bacterial community stable.

The source of samples or the original microorganisms prior to the enrichment and the enrichment conditions have great influence on the community and function of

MMCs. Van der Ha et al. (2010) enriched MMCs from effluents of anaerobic digesters and found that adding copper increased the activity as well as salt resistance of the cultures since the activity of pMMO was enhanced. Pfluger et al. (2011) inoculated Type II *Methylocystis*-like dominated mixed culture in a fluidized bed reactor. Elevating dissolved oxygen (DO) and using nitrate as N-source shifted the dominant methanotroph to *Methylobacter*-like Type I without polyhydroxybutyrate (PHB) production. The key selection factors favoring Type II methanotrophs as well as PHB production were low DO and nitrogen as N-source. Chidambarampadmavathy et al. (2015, 2016) enriched MMCs from soils of landfill and compost, respectively. Although the predominant bacteria were *Methylosarcina* sp. and *Chryseobacterium* in both cultures, the characteristics for PHB production were different. With variable methane to oxygen ratios, the methane oxidation capacities and PHB content of the cultures fluctuated, but the community structures were relatively stable.

The microorganisms in MMCs not only rely heavily on the richness and diversity of the original sources but also are sensitive to some culturing factors. In addition, the requirements for growth and function of MMCs are sometimes competitive or even conflicted. Therefore, the enrichment and cultivation strategies of MMCs should be considered and designed in conjunction with the application objectives from the beginning.

Different from enriched MMCs, directly mixing several kinds of isolated or mixed MOB usually cannot achieve the positive effects. For example, the mixture of *M. trichosporium* OB3b, *M. trichosporium* IMV3011, *M. capsulatus* HD6T, and *Methylomonas* sp. GYJ3 did not show advantages in the activities of propene epoxidation and naphthalene oxidation, and the ability of PHB synthesis, comparing to the respective pure methanotrophic cultures (Zhang et al. 2009). Pieja et al. (2012) subjected the mixture of *M. parvus* OBBP, *M. trichosporium* OB3b, *M. hirsuta* SV97, *Methylocystis* 42/22, and an enriched culture containing primarily the first three species to cyclically stressed conditions. PHB production increased only in the reactor limited by nitrogen and methane, which was dominated by *M. parvus* OBBP with no detectable minority populations. That is, the other initially coexisting bacteria had been eliminated during the stressed cultivation. These results are not surprising, because the knowledge of individual methanotrophs and the interactions with other bacteria are limited. It is impossible to carry out a rational design of a synthetic MMC without big data.

Enrichment and acclimation are time-consuming processes. Once the cultures of stable MMCs are acquired, a preservation method should be considered. Placing the culture at 4 °C and refreshing substrates as well as nutrients regularly can preserve the community for a short period. If a large number of cells were collected by centrifugation, they can be directly cryopreserved at −80 °C for a relatively long time. In order to avoid cellular damage during cryopreservation and subsequent unfreezing, it is necessary to add a cryoprotective agent. Paraffin oil (Jiang et al. 2014), dimethyl sulfoxide (DMSO) and DMSO plus trehalose and tryptic soy broth (TT) (Kerckhof et al. 2014) were proven effective for cryopreservation of MMCs. After preservation for several months, the activity recovery was fast, and the stable community structure was maintained.

Besides obtaining cultures ex situ, stimulating or incubating the indigenous MOB for in situ application is also a common approach. Taking bioremediation of the groundwater contaminated with trichloroethylene (TCE) as an example, the nutrients of methane, oxygen, nitrate, and phosphate (Eguchi et al. 2001) as well as electron donor (Conrad et al. 2010) were introduced for bio-stimulation. The methane oxidation with increasing MOB and the TCE degradation were observed.

It is worth noting that different kinds of MOB have distinct functional features and may render a selective advantage under different conditions (Ho et al. 2013). Some hypotheses have been proposed for ecological characteristics and life strategies of Type I and Type II methanotrophs, according to their performances in environments. Type I methanotrophs are more prevalent in areas with low methane (0.1–0.3%), while at high methane ratios, Type I and Type II methanotrophs tend to contribute equally to methane metabolism (Henckel et al. 2000; Jiang et al. 2016b). Using nitrogen as N-source preferentially selects Type II methanotrophs, although the potential for nitrogen fixing ability is found in other MOB (Conrad 2007; Trotsenko and Murrell 2008). Ho et al. (2013) put forward a competitor-stress tolerator-ruderal functional classification framework to illustrate the life strategies among Type I and Type II MOB, where Type I is broadly classified as competitor-ruderal, while type II fits more within the stress tolerator categories. Although these hypotheses cannot be suitable for all situations and do not have sufficient theoretical supports, they still provide helpful insights for acquisition, optimization, and prediction of MMCs.

15.3 Interactions of Methanotrophs with Coexisting Heterotrophs

Through cooperation of methanotrophs and heterotrophs, MMCs can achieve better growth, generate bioproducts, and actuate processes. Therefore, the relationship and interaction among them is an interesting and important topic.

MMCs are generally methane-driven systems, where methanotrophs utilize the primary substrate methane to grow and supply metabolites as subsequent feed for the associated heterotrophs. In return, the coexisting heterotrophs can promote methanotrophic growth and activity by removing the toxic metabolites or producing beneficial additives. For example, cobalamin excreted by rhizobial strains was found to have great positive effects on some gammaproteobacterial methanotrophs (Iguchi et al. 2011). In industrial production of SCP, the producer *M. capsulatus* Bath may benefit from association with the heterotrophic partners, as they can avoid accumulating toxic levels of acids in the culture (Bothe et al. 2002).

The microbial interactions within MMCs are much more than the exchange of metabolites. Hršak and Begonja (2000) investigated a methanotrophic-heterotrophic groundwater community in the presence of methane as the primary carbon and energy source and linear alkylbenzene sulfonate (LAS) as a co-metabolic substrate. The growth of methanotroph was stimulated or inhibited while co-culturing with individual heterotrophs, but it was not affected by their lysates. Furthermore, the

capacity of LAS degradation in a descending order was the original six-member community, two-member reconstructed communities, and isolated methanotroph. Ho et al. (2014) assembled artificial communities with a model methanotroph of *Methylomonas methanica* and ten heterotrophic species. In the early phase of the logarithmic growth, methane oxidation significantly increased with the increasing heterotroph richness, suggesting an interaction leading to a stimulation of methanotrophic activity. We also found that MMO activity and methane oxidation of a culture with more heterotrophic richness appeared higher in the early growth stage but became lower later; in a whole growth period, this more diverse consortium had no obvious advantages in terms of methane oxidation (Jiang et al. 2014, 2016b).

In MMCs, methanotrophs and the coexisting heterotrophs carry on a variety of actions and interactions, forming a huge, multi-role, and dynamic ecological network. If enough data were obtained, it may be possible to employ tools and approaches of ecoinformatics to deal with this complex system. In the view of ecoinformatics, ecology is seen as a data-driven science, and the data can be transformed into information and knowledge (Michener and Jones 2012). Stock et al. (2013) incubated 9 methanotrophs with 25 heterotrophic strains in a pairwise miniaturized cocultivation setup and proved that it would be possible to construct data-driven tools to predict methanotrophic-heterotrophic interactions. It is a proof of principle with a small dataset. However, following the continued exploration and deeper understanding of MOB, larger datasets containing more methanotrophs and heterotrophs will be achieved, and more links of these data with genetic and metabolic information can be revealed. These advances will promote the availability of the statistical approaches.

15.4 Applications of MMCs in Environmental Bioengineering

Appling MOB in environmental bioengineering can be divided into two categories as methane mitigation and co-metabolism of other pollutants and as in situ bioremediation in fields and ex situ biodegradation in bioreactors. Ex situ processes for the removal of contaminants have two forms of configurations, single-stage and multistage, based on the separation of bacterial growth/recovery and contaminant degradation or not. The robust and multifunctional MMCs are an appropriate choice to apply in this area.

Within the methane emissions caused by human activities, landfills are one of the most important sources and relatively easy to control. Previous reviews have comprehensively summarized relevant aspects about the elimination of methane from landfills, such as mechanisms, factors, modeling, processes, lab studies, field trials, and challenges (Chiemchaisri et al. 2012; Huber-Humer et al. 2008; Nikiema et al. 2007; Sadasivam and Reddy 2014; Scheutz et al. 2009). The indigenous microorganisms are mostly employed, and biofiltration is the main approach or concept. The knowledge and experience obtained from the treatment of landfill methane can also be extended to other methane mitigation, particularly applicable to the emissions where methane is collectible.

Owing to the presence of a broad-spectrum MMO, MMCs can be utilized for the degradation of organic contaminants like alkanes, alkenes, alicyclic hydrocarbons, halogenated aliphatics, etc. Some recent studies are listed in Table 15.2. One of the research hotspots is using MMCs to remediate soil or water contaminated by halogenated hydrocarbons like TCE. Smith and McCarty (1997) found that methane addition to the transformation stage inhibited TCE co-metabolism at low TCE concentrations and enhanced it at high TCE concentrations, suggesting that TCE co-metabolism in the presence of growth substrate does not simply follow the competitive inhibition kinetics.

sMMO is generally considered to be the better enzyme for contaminant degradation due to the broader substrate range, and indeed some MOB expressing sMMO show excellent ability to co-metabolize different kinds of contaminants. However, pMMO have a much higher specificity for methane, and the MOB expressing pMMO are supposed to be more robust against the toxic metabolites (Anderson and McCarty 1997; Benner et al. 2015). Some MMCs expressing pMMO have equal or even better ability for degradation of certain pollutants. With copper addition to induce pMMO expression and repress sMMO, Benner et al. (2015) indicated that co-metabolic degradation of the aromatic compounds sulfamethoxazole (SMX) and benzotriazole (BTZ) was possible by pMMO; Anderson and McCarty (1997) found that the transformation yields for *trans*-1,2-dichloroethylene (t-DCE) and vinyl chloride (VC) by MMC expressing pMMO were 20 times greater than the yields reported by sMMO-expressing cells.

If MMCs are combined with other biotic systems, the applications will be greatly expanded. For example, biodegradation of chloroethenes can be performed sequentially by reductive dechlorination under anaerobic conditions and oxidative degradation, where the disadvantages of reductive dechlorination are overcome and complete mineralization of the chlorinated pollutants is achieved (Tiehm and Schmidt 2011). Usually, the anaerobic and aerobic degradation occurs in two separated phases, but some researchers coupled the anaerobic and aerobic catabolisms of methanogenic/methanotrophic consortia in a single system (Guiot et al. 2008; Tartakovsky et al. 2003). Aerobic methane oxidation coupled to denitrification (AME-D) links the functions of aerobic methanotrophs and denitrifiers, which have potential for nitrogen removal from wastewater, drinking water, and groundwater (Zhu et al. 2016). Sun et al. (2013) performed AME-D in a membrane biofilm reactor, where methane and oxygen were provided by a polyvinylidene fluoride membrane for biofilm. With the synergistic action of methanotrophs and denitrifiers, up to 97% NO_3^-–N was removed. Van der Ha et al. (2011) combined a MMC with microalgae in sequence batch reactors and yielded more biomass with 55% less external oxygen supply, as oxygen was produced in situ by the microalgae. Almost all carbon dioxide produced by MMC was converted to biomass by microalgae. Similarly, a mutualistic symbiosis between MMCs and brown mosses reduced methane emissions from Arctic polygonal tundra by at least 5% (Liebner et al. 2011).

Table 15.2 Summary of recent researches about pollutants degradation by MMCs

MMC	Pollutants	Reactors and processes	Results	Reference
MMC enriched from aquifer material	HCFCs and HFCs	Batch culture in bottles with separated growth and degradation	Order of reactivity HCFC-22 > HCFC-142b > HFC-t34a > HCFC-123, with second-order rate coefficients of 0.014, 0.0096, 0.00091, and 0.00054 L/mg day, respectively. T_c 0.0025 g HCFC-22/g VSS and 0.0011 g HCFC-142b/g VSS	Chang and Criddle (1995)
MMC expressing pMMO	t-DCE, VC, c-DCE, TCE, and 1,1-DCE	Batch culture in bottles	T_y3.0 g t-DCE/g CH_4, 0.79 g VC/g CH_4, 0.31 g c-DCE/g CH_4, 0.014 g TCE/g CH_4, and 0.0012 g 1,1-DCE/g CH_4	Anderson and McCarty (1997)
MMC enriched by conditions of enhancing sMMO expression	TCE and/or cDCE	Culture growth in a CSTR and contaminant degradation in a PFR	Treating wastewater containing TCE (4.7 mg/L) and cDCE (4.8 mg/L) to below 5 μg/L each continuously for at least 31 days. T_c 0.53 g TCE/g VSS and 0.55 g cDCE/g VSS	Chang and Alvarez-Cohen (1997)
MMC included Type II species	TCE	Culture growth in a CSTR and contaminant degradation in a PFR	T_c 0.088 g TCE/g VSS, T_y0.026 g TCE/g CH_4	Smith and McCarty (1997)
Indigenous microorganisms	TCE	Methane, oxygen, nitrate, and phosphate introduced into groundwater contaminated with TCE	TCE removal 10–20%, T_y 0.003–0.013 gTCE/gCH_4. After methane injections stopped, no TCE removal observed	Eguchi et al. (2001)
MMCs from five different inocula	SMX and BTZ	Batch culture in bottles with growth and degradation together	After 7 days, SMX removal 90–100%, BTZ removal 50–76%	Benner et al. (2015)

VC vinyl chloride, *1,1-DCE* 1,1-dichloroethylene, *cDCE cis*-l,2-dichloroethylene, *t-DCE trans*-1,2-dichloroethylene, *TCE* trichloroethylene, *HCFCs* hydrochlorofluorocarbons, *HFCs* hydrofluorocarbons, *SMX* sulfamethoxazole, *BTZ* benzotriazole, *CSTR* continuous stirred-tank reactor, *PFR* plug flow reactor, *VSS* volatile suspended solids, T_c transformation capacity, T_y transformation yield

15.5 Applications of MMCs in Biocatalysis

Due to the cooperation of various bacteria and enzymes in MMCs, the degradable metabolites are not easy to accumulate. Therefore, many bioproducts that can be generated by isolated methanotrophs will not be observed in the culture of MMCs. But the production of SCP and biopolymer is not affected.

In the 1980s, Larsen developed the commercial production of methanotrophic SCP in Denmark, and based on this technology, the company Unibio was founded in 2001 (Strong et al. 2015; Unibio 2016). Now SCP is a successful and commercial representative of bioproducts generated by MOB using natural gas as methane source. Besides methane, different concentrations of ethane, propane, and higher alkanes may be contained in natural gas, which will cause accumulation of acetate and propionate in the culture. Bothe et al. (2002) found that three heterotrophic bacteria consistently contaminated *M. capsulatus* in long period cultivation under semi-sterile conditions. They were *Aneurinibacillus*, *Brevibacillus agri*, and *Ralstonia* strains, which helped to remove the inhibiting metabolites for the methanotrophs.

Another product generated by MMCs and may have commercial prospect in the near future is PHB. As the first discovered and most studied polyhydroxyalkanoate (PHA), PHB is a promising biopolymer for substituting petroleum-based plastic. The current knowledge and highlight research about methanotrophic PHB/PHA production from methane are summarized in detail in some reviews, including applying MMCs as the producers (Karthikeyan et al. 2015; Khosravi-Darani et al. 2013).

PHB can be accumulated in both Type I and Type II MOB, but Type II methanotrophs using the serine pathway of carbon assimilation are more effective (Wendlandt et al. 2001). The common PHB producers of Type II include *Methylocystis paravus*, *Methylosinus trichosporium*, *Methylosinus sporium*, and *Methylocystis* spp. (Khosravi-Darani et al. 2013).

Wendlandt et al. (2010) developed a robust and economical two-stage process to produce PHB, using a MMC dominated by *Methylocystis* sp. GB 25 ($\geq$86% of total biomass) in an open system. Pressure bioreactor was developed to enhance mass transfer of methane and oxygen into the liquid growth medium. Since PHB is usually accumulated as a carbon/energy or reducing power storage material when a limitation of growth components exists, the synthesis greatly depended on the applied nutrient deficiency. The best results were achieved when nitrogen was depleted, where the maximum biomass concentration and polymer content were 60 g/L and 51%, respectively, after 24 h.

An algal *Scenedesmus* sp. and *Methylocystis parvus* were co-cultured with biogas as substrate to harvest bioflocs in two- and one-stage processes, respectively (van der Ha et al. 2012). Without external oxygen provision, N-limited growth was chosen to enhance lipid production by algae and PHB accumulation by MOB, and 98% of consumed CH_4–C as well as CO_2–C was found back as biomass. PHB yield achieved 295 mg/g cell dry weight by repeated N-limitation.

Till now, most reported studies have been performed in small serum bottles, and the conclusions need to be further verified and corrected in pilot-scale tests. And according to theoretical calculations, the PHA/PHB accumulation in Type II methanotrophs can be as much as 0.68 g/g dry biomass (68%) (Karthikeyan et al. 2015); however, the experimental results usually cannot reach it. Levett et al. (2016) presented a techno-economic assessment by ASPEN Plus for production of 100,000 t/a PHB through methanotrophic fermentation and acetone-water solvent extraction. The costs were estimated to be $4.1–$6.8/kg PHA, and heat removal from the two-stage bioreactor process took 28% of the operating cost.

To enhance competitiveness of the methanotrophic PHA/PHB compared to the petrochemical products, more effort is needed to improve the properties of the biopolymers and reduce the cost.

15.6 Conclusion

The MMCs made up of MOB and other coexisting microorganisms not only possess the unique characteristics of methanotrophs like methane assimilation and broad substrate range but also can overcome the shortcomings in pure methanotrophic cultivation like slow growth rate, low cell density, and poor MMO stability. Coordinated with other functional systems, MMCs may further extend their application scope and field.

Figure 15.1 gives a schematic diagram of acquisition and application of MMCs. Currently, a complex MMC is in general still like a black box. On the one hand, the understanding of the individual microorganism acting in the consortium is limited, especially the knowledge of MOB. On the other hand, little is known about the microcosmic interactions and influence mechanisms among the methanotrophs and

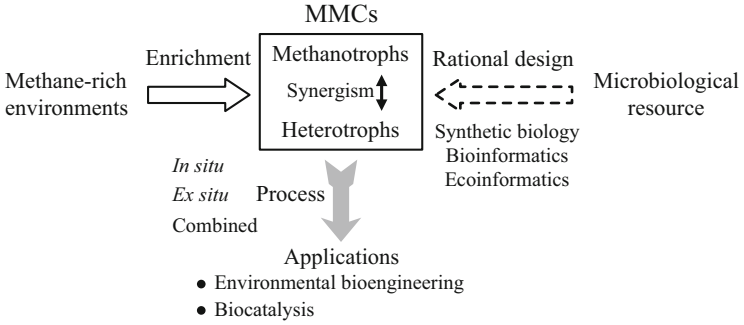

Fig. 15.1 Schematic diagram of acquisition and application of MMCs. With the biochemical, genomic, and proteomic analyses of methanotrophs, molecular level of metabolisms may be recognized someday. Genetic tools and synthetic biology provide possibilities to essential and persistent improvement of their capabilities. And the development of ecoinformatics will help to describe the complicated interactions in MMCs. Rational design of MMCs and other achievements can be expected by these advances and efforts

coexisting heterotrophs. Even though some regularities are frequently observed, exceptions are common. These are all challenges that need to be faced with in the theoretical research and practical application of MMCs.

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Environmental Life Cycle Assessment of Methane Biocatalysis: Key Considerations and Potential Impacts

16

Robert M. Handler and David R. Shonnard

16.1 Introduction

Environmental impacts associated with production and use of all industrial products are becoming a matter of increasing concern. In particular, emissions of greenhouse gases from industrial processes and transportation are a primary concern that is driving a great deal of regulatory action around the world. The California Low Carbon Fuels Standard (CARB 2009), the US EPA Renewable Fuels Standard (U.S. EPA 2009), and other regulatory systems are supplying pressure to develop fuels with superior environmental benefits and offering financial incentives to produce fuel products with low global warming impacts. Regulations governing the chemical industry [e.g., REACH in European Union (2006)] are also prompting further development of chemicals with lower environmental impacts. Product trade organizations and companies are committing to reduce the greenhouse gas emissions associated with their products and potentially increase market share or respond to regulatory pressures as well (e.g., Cosmetics Europe 2012; Dow Chemical Company 2016).

The emphasis on lowering environmental impacts of products, combined with a recognition that impacts can occur over the entire product life cycle of raw material procurement, material conversion, supply chain activities, product use, and product end of life, requires a comprehensive method of tracking and quantifying impacts. Environmental life cycle assessment is such a method, with well-developed methods established according to International Organization for Standardization (ISO) that are

Prepared for a chapter in the volume, "Methane biocatalysis: paving the way to sustainability", edited by Marina Kalyuzhnaya and Xin-Hui Xing

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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_16

still flexible enough to allow application in a wide variety of areas, from renewable fuels to impacts associated with chemicals, durable goods, or other areas (ISO 2006).

Rapidly developing sets of life cycle inventory databases, such as ecoinvent (Weidema et al. 2013) or the US LCI database (NREL 2012), catalog the inventory of inputs and outputs associated with the production of an ever-increasing set of materials, energy sources, and processes such as material transport or chemical refining that are needed to represent a full life cycle of a new product. This focus on environmental impacts over the entire life cycle of a product can encourage wise use of resources, focus research on key areas of environmental impact within the product life cycle, and development of new strategies for supplying services with lower environmental impacts.

Methane gas is a valuable chemical resource, both as a source of energy and as a chemical feedstock. Global methane use is increasing, in part due to our ability to access methane resources once deemed to be technically unavailable, through new production methods like hydraulic fracturing. Increasing attention is being paid to stranded methane, which can be defined as methane from sources that are unconnected to conventional methane collection and processing infrastructure, due to economic, technical, or political considerations. For example, a gas-producing facility may be located a large distance from existing pipelines or from likely processing facilities, or the methane resource may have a limited quality or projected volume, such that investment in pipeline infrastructure is too challenging.

Even though the volume of stranded gas available at a specific location may be low, the total volume of methane that might be considered as stranded is actually quite large in aggregate (Attanasi and Freeman 2013). There is also great potential to reduce environmental impacts associated with stranded methane resources. Methane is a potent greenhouse gas, with a 100-year global warming impact over 20-fold greater than CO_2 (IPCC 2013). Due to the lack of economic interest in stranded gas reserves, methane is often flared or released directly into the atmosphere at the stranded gas location. The ability to avoid this harmful flaring or release activity and also create useful products from the available methane resources makes increasing attention on stranded methane resources a worthwhile endeavor.

Methane biocatalysis is one promising strategy for utilization of stranded methane, with the ability to make a wide range of bio-based chemicals and fuel products. Biocatalytic processing methods have great potential to take advantage of stranded methane sources because bioreactors may be deployed at a suitable scale, with lower infrastructure impacts and capital costs that are suited to the lower potential lifetimes of specific methane sites. Not every use of stranded methane is likely to have positive environmental outcomes relative to comparable fuels or chemicals already on the market, however. A careful consideration of the environmental impacts at all stages of the new product life cycle will be necessary to ensure that the new products that are created from these resources are environmentally preferable to current conventional products.

In this chapter, environmental life cycle considerations associated with methane biocatalysis to produce fuels and chemicals are reviewed. A few example scenarios are also presented as case studies to explore the relative impacts of different

processing decisions across the product life cycle and highlight the importance of key assumptions in the system.

16.2 Overview of Methane Biocatalysis Life Cycle Assessment Considerations

Many possible options exist for procurement and utilization of stranded methane resources. To discuss the general considerations associated with methane biocatalysis, this chapter will consider a general product life cycle composed of distinct unit operations, as diagrammed in Fig. 16.1. Not all of these unit operations may be required for every possible biocatalysis process, and some of these unit operations may be accomplished using technologies that involve a unique set of inputs. This discussion will focus on the general considerations at each life cycle stage and the typical inputs of materials or energy that would likely be required at each stage, using technology that has been proven in other systems. Awareness of the life cycle inputs required for production of a given amount of biocatalysis product will be critical for developing a full life cycle inventory of inputs and outputs for the process. The full life cycle inventory can then be translated into a comprehensive picture of the life cycle environmental impacts, according to well-established and clearly documented impact assessment methods, such as the Intergovernmental Panel on Climate Change (IPCC) 100-year global warming potential. Each general unit operation is discussed in more detail in the following sections.

16.2.1 Methane Feedstock Procurement

As discussed above, stranded methane resources may be currently stranded for several reasons related to economic or technical constraints that have prevented integration of the methane source with traditional large-scale methane collection and processing pipeline infrastructure. If the methane reserve is in a remote region of the world, or located out in the ocean, it may be too costly to connect to the existing pipeline network. Some methane sources, such as landfills, may have gas outputs that are too short-lived or have a methane concentration that is too low to justify the costs of conventional pipeline connection. There may also be other considerations preventing traditional use of the methane such as technical or political constraints.

Gas that is produced along with conventional oil production is commonly referred to as associated gas. It often has a low market price and production volume relative to the oil that is produced on-site, and the gas may have low overall methane content; therefore the development of the site may not focus on maximizing the capture of the associated gas. Previous Department of Energy-sponsored investigations from collaborators have determined that associated gas often has a 30-fold lower economic value than the oil extracted at a given site (Ceres 2013). Even when pipeline infrastructure is in place to capture associated gas, limited pipeline capacity at the site combined with variable production of the associated methane gas often results in

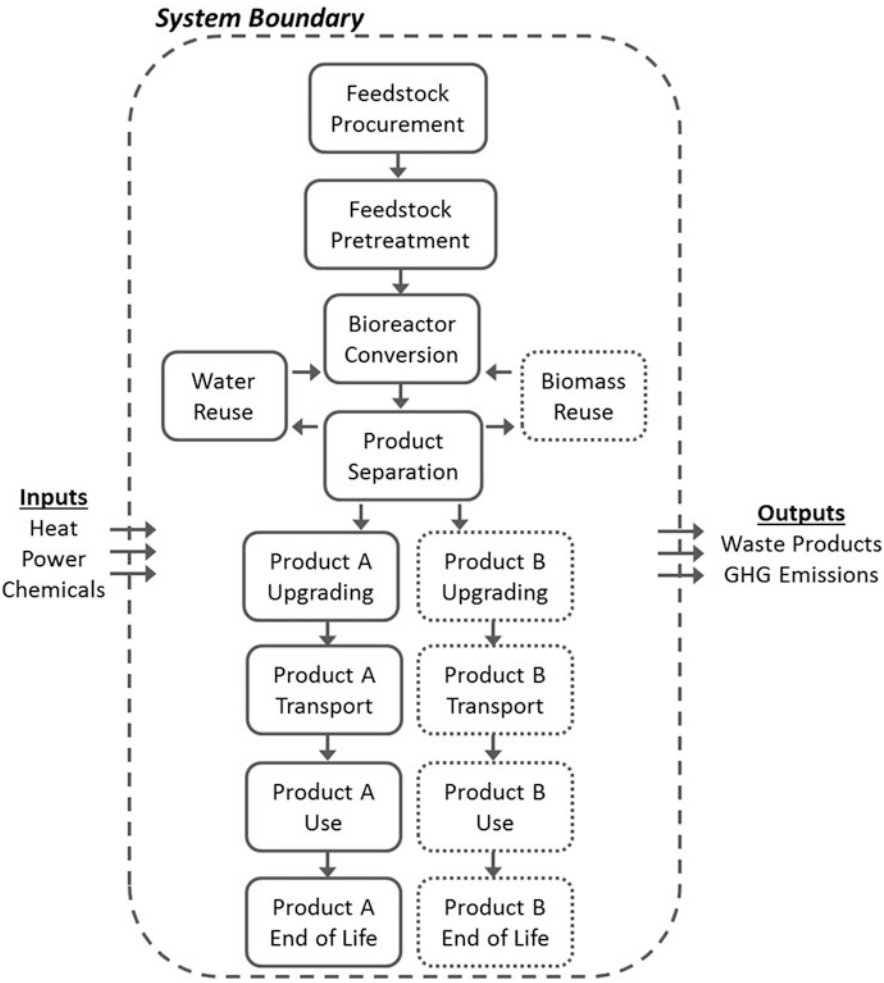

Fig. 16.1 Schematic of general methane biocatalysis product life cycle. The short dashed lines for unit operations associated with Biomass Reuse and the Product B downstream supply chain indicate that these operations are optional for the purposes of this general overview. They are evaluated as scenarios in this chapter

situations where the associated gas is stranded for all practical purposes because it cannot be captured and there are no storage systems in place.

Due to situations like this, it is not uncommon for stranded methane gas sources to be vented directly to the atmosphere or flared to combust the methane into CO_2 before emission to the atmosphere, resulting in a large release of greenhouse gases without any productive benefit. Taking this wasted carbon resource and instead routing it through a biocatalytic process would avoid the large impacts associated with flaring or otherwise releasing the gas into the atmosphere and convert the gas

into a higher energy-dense form that may be economical to transport. From a product life cycle perspective, this avoided flaring would result in a credit to the product system for avoided environmental impacts. Alternatively, if the methane gas was already being collected and routed for a productive use, it would not be considered a "stranded" resource, and there would be no flaring of the methane gas to avoid by introducing the bioprocessing system into the site, and consequently there would be no credit to the system for avoiding the flaring and release carbon-containing gases to the atmosphere at this stage of the process. As an added complexity, there are even situations where the methane gas is already providing a productive service at the site of extraction and collection. For instance, associated gas is often pumped back down into oil wells to increase pressure and improve oil recovery, or it may be burned on-site to provide useful heat and power. In situations like these, it may be appropriate to consider how these useful services might be replaced at the site if the methane-containing gas was instead diverted to the biocatalytic process. The impacts associated with providing that useful service (power, heat, or pumping capability) would therefore need to be accounted for in the new product life cycle. For this reason, when focusing on the gas procurement stage of a life cycle assessment in this system, it is important to determine the likely alternative fate of the stranded resource or model a few likely scenarios to capture the full range of potential environmental outcomes.

To utilize this stranded resource, it may be necessary to construct a limited amount of pipeline infrastructure to collect and transport methane, perhaps linking several associated gas wells in a nearby area to a centralized bioprocessing facility. The large initial impacts associated with investing in the collection infrastructure (energy, materials) can usually be considered to be attributed to all of the methane that will be produced at the site over the productive lifetime of the site, so the overall impacts associated with infrastructure development normalized per unit of methane gas are typically quite low.

In areas where oil exploration is expanding rapidly, in remote areas of the world, or in areas without strict environmental safeguards or access to capital, there are clearly opportunities for a low-cost alternative methane utilization strategy to avoid the mismanagement of this stranded gas. Depending on the particular circumstances surrounding the specific stranded gas resource, incorporation of a biocatalytic methane processing technology may provide a viable alternative.

16.2.2 Methane Feedstock Pretreatment

At this stage of the product life cycle, the methane-containing gas may need to undergo some pretreatment or processing before biocatalysis. This may involve the removal of impurities through physical or chemical methods, if the gas contains substances that could interfere with downstream unit operations. For instance, compounds with sulfur or metals may inhibit growth of organisms in the bioreactor, which would require removal to achieve optimal performance. Purification systems may involve the input of utilities, chemical sorbents, or other items at this stage.

The methane-containing gas stream will need to be pressurized, as will other input gas streams that are needed for bioreactor operation. Power requirements for pressurization through multistage compressors may be high, depending on the performance characteristics of the bioreactor, which will dictate the mass transfer of gas into the bioreactor media. Methane has a very limited solubility in aqueous-phase media across a range of potential pH values, which means that the performance of the biocatalysis process will likely be limited by mass transfer of methane gas into the aqueous media. One way to functionally increase methane mass transfer is to force a large amount of methane gas through the media under high pressures, but this is an expensive and inefficient method of increasing mass transfer. Due to this large power requirement for input gas pressurization, it may be worth exploring the feasibility of adding systems to purify the input gas streams prior to the bioreactor stage. If the input gas stream has a low concentration of methane, gas purification technologies like pressure-swing absorption may be used to concentrate methane in the input gas stream prior to the bioreactor stage, which would then require compressing and moving less gas through the bioreactor to introduce the same quantity of methane. In this way, process engineers would have to assess the trade-offs that would be required between the inputs required for operating the purification systems (power, chemicals) and the reduction in power requirements for compression that would result from working with a higher-purity input gas.

16.2.3 Methane Bioconversion

At this stage of the product life cycle, biocatalysis of the methane occurs inside a specialized reactor. Several organisms are being isolated for methane utilization (Strong et al. 2016), and many candidate organisms can potentially be genetically modified to improve performance through increasing conversion efficiencies, increasing tolerance to impurities, or changing other characteristics such as pH tolerance to optimize overall performance. In general, bioreactor operations will require power for mixing and pumping requirements and additional power or steam for reactor cooling or heating to maintain optimal performance, which may change over the course of a year in different operating conditions. All of the necessary macronutrients and micronutrients for organism growth and biocatalysis will need to be supplied, often in excess of stoichiometric requirements due to incomplete utilization. Additional chemical additives may also be needed to achieve specific solution requirements, related to removing impurities, maintaining system pH, viscosity, or other operating characteristics. Makeup water will also be needed to replace water lost from outputs leaving the bioreactor system, even if most of the water is ultimately recycled after product separation.

An area of active research related to this stage of the life cycle is to develop reactor systems with improved gas transfer properties. This would reduce the power requirements associated with gas compression in the preceding pretreatment operations discussed above, due to low gas pressures required to achieve satisfactory levels of mass transfer in such a bioreactor with improved mass transfer. The

conventional standard reactor design for this heterogeneous gas–liquid system is a bubble column, where input gas is introduced in the bottom of a reactor vessel containing the aqueous phase, causing gas to bubble up through the reactor and exit from vents at the top of the reactor. During the transit of gas bubbles from the bottom of the reactor to the top of the reactor, gas molecules have the opportunity to transfer into the liquid phase at the interface of the gas bubble and the aqueous media. A great deal of research has explored this conventional reactor system in an effort to increase the mass transfer properties of the gas to liquid exchange by optimizing the geometry of the reactor components or making other modifications. For instance, adding a new technical component like low-frequency vibrations was observed to increase mass transfer properties in a similar system by 50–100% (Krishna and Ellenberger 2002). In another recent effort, LanzaTech led an ARPA-E REMOTE project with Michigan Technological University and other partners to optimize gas fermentation systems through a variety of biological and engineering improvements. The REMOTE program objective was to develop reactor systems which increase mass transfer properties by several-fold, compared to standard bubble column systems. The impacts of improvements in this area on upstream gas pretreatment requirements are a useful illustration of the need for a comprehensive view of the whole product life cycle when evaluating the impacts of potential changes to a complex product system.

16.2.4 Product Separation

During the preceding bioreactor conversion stage, methane will be utilized to produce something of value using a biological process or possibly even multiple products. Depending on the nature of the desired product or products, the product separation stage will actually involve several related unit operations, with careful coordination between the separate steps. If the products from bioconversion are excreted from the organism, the product may simply need to be isolated from the aqueous phase and purified via distillation or other means. If the product or products are created and stored inside the growing organisms of the bioreactor, the biomass will need to be harvested from the bioreactor, using simple unit operations such as flocculation, dissolved air flotation, or membrane systems to concentrate the biomass in a downstream operation. Additional steps may be needed to remove the target product and/or additional water from the biomass stream in order to proceed to product purification, which may be accomplished with centrifugation or evaporation with the addition of thermal energy. The extent of water or product removal will be dependent on the specific products in question and the requirements of useful product separation technology. For instance, lipids may be separated from cellular biomass after the solids content has reached at least 20%, using a combination of cell fractionation and hexane extraction steps (Frank et al. 2011). Other products may require different stages of chemical or physical processing, and the inputs of utilities or other requirements will have to be included in the product life cycle.

If multiple products (e.g., Product A and Product B in Fig. 16.1) destined for external use are created in the methane biocatalysis system, the proper allocation of process inputs and ultimate environmental impacts, between different products, will need to be considered. At this point in our consideration of a general methane biocatalysis system, many inputs to the system have been required to satisfy all of the gas procurement, gas pretreatment, bioconversion, and product separation activities. All of the inputs and outputs of these "shared" unit operations that are common to both products will result in environmental impacts, and the impacts of these "shared" unit operations should be shared between the products. After this product separation stage, any processing requirements or environmental impacts associated with further development and use of Product A should be attributed solely to Product A, because those unit operations are not relevant to the life cycle of Product B anymore. There are many strategies for allocating "shared" impacts between different products, and applying two different allocation strategies to the same system will result in a different distribution of impacts between coproducts. It is therefore important to be transparent about which allocation system is being used to distribute impacts in a given system. Environmental impacts can simply be allocated on the basis of mass. For example, a system which produces 10 kg of Product A and 90 kg Product B will allocate the impacts of shared operations by assigning 10% of the impacts to Product A and 90% to Product B. If the products are energy carriers (e.g., a biofuel process that creates gasoline and propane), it may be more appropriate to allocate the impacts based on energy content of the products, such as the total MJ of Product A versus total MJ of Product B. Others have argued that economic considerations ultimately govern the decisions to produce and collect multiple products in a system, so economic allocation (total economic value of Product A vs. Product B) is the most appropriate allocation scheme. Another common method of allocating environmental impacts is system expansion and displacement, where all of the impacts for the combined product system are allocated to Product A, but a credit is given to the system on the basis of the avoided production of Product B, since any units of Product B that are created in this new product system will displace units of Product B that are produced through other means. For instance, an ethanol system that produces ethanol and distiller's grains may use a system expansion and displacement method to allocate all environmental impacts to ethanol production but claim a credit for distiller's grain production on the basis of those grains displacing the need for other animal feed products. Different regulatory bodies will encourage or require the use of different allocation systems, so it is important to understand the implications of these systems on the ultimate outcome of the life cycle assessment.

At this stage of the methane bioprocessing system, it is also important to consider that one "product" may be the residual biomass that is left over after the desired products have been removed from the biological system. For instance, if lipid production is the primary target of the process, there will still be a large quantity of non-lipid biomass remaining after lipids have been extracted and sent to further processing steps. This non-lipid biomass may have several potential fates. As a product for external markets, it may have value as a food source for shrimp or as a

fertilizer. It may also be recycled and used internally to recover a portion of the nutrients and energy content that was already invested to initially produce the biomass, which would reduce the need for external inputs of chemicals or utilities. If there are no external markets for extra biomass, or if the technical complexities of system integration are too great in certain situations, this extra biomass may simply have to be treated as a waste product that is created from the product system. After product separation, an opportunity may also be available to recycle a portion of the aqueous phase back into the bioreactor, as a way to recycle process water and some bioreactor chemicals.

16.2.5 Product Transport

After products have been separated, they will likely require transportation from the location of the methane bioreactor to locations where they may be further processed or ultimately used. Truck transport is most commonly assumed for production of fuels and chemicals, although rail transport may be available depending on the particular site of a stranded methane resource. As an initial exercise, specific transport distances between bioprocessing sites and ultimate product use sites may not be well characterized, or products may be sent to a variety of potential use sites, so generalized transport inputs representing average transport around a particular region may be used, but these assumptions should be revisited as specific project opportunities are developed and studied.

16.2.6 Product Use

Depending on the products, the use phase of the product may be short or extend over several years, with more inputs of materials or energy and significant environmental impacts, or may be a relatively unimportant phase of the overall life cycle. For a fuel product, the use phase involves combustion and release of gases to the atmosphere. If products were intermediates to plastics or other stable chemical products, further processing may be required, but the product may have a long and relatively benign use phase. Consumable chemicals may have a variety of further processing requirements before use, and their use phases may be quite variable in practice, with a variety of potential associated inputs and outputs.

16.2.7 Product End of Life

Similar to the product use section above, the impacts associated with the end of life is highly dependent on the specific nature of the product. For an energy carrier like a liquid fuel, the product has already been combusted during the use phase, so there are no extra additions to the life cycle at this stage. Plastics may be either recycled to create new products, or disposed of in a solid waste landfill, so the inputs and outputs

to the environment, including the new service offered by recycled goods, would need to be considered. Consumable chemical products may ultimately be disposed of and make their way to a wastewater treatment system before release to the environment. Assumptions made at this stage concerning likely outcomes should ideally be made based upon the known fates of comparable products, or identical products made through different production systems, but these assumptions should all be documented and assessed to see if they reflect likely outcomes for this new product system as well.

16.3 Case Study to Illustrate Impacts of Different Scenarios

As we can see from the brief descriptions of generalized unit operations above, the overall process of methane biocatalysis to form useful products is complex, with several steps, and many embedded assumptions and potential options for executing the specific steps. In an effort to paint a comprehensive picture of the potential LCA outcomes of the full process and assess the impacts of a few alternative scenarios at each stage of the process, the set of potential scenarios to evaluate becomes quite large. As part of a Department of Energy-funded project evaluating the impacts of 1 methane utilization technology, we have collaborated with others to evaluate well over 100 life cycle systems, comprising different combinations of options for methane procurement, gas pretreatment, bioconversion, product separation, and product use.

To present a simple illustration of the impacts of different choices that can be made at each stage on the overall life cycle outcomes for this system, a very simplified case study is presented below. A brief description of assumptions and input data for key life cycle stages is presented, followed by an overview of LCA results for the scenarios and a discussion of lessons learned.

The set of five scenarios presented below is summarized in Table 16.1. To simplify the presentation, we are only focusing on a small subset of potential decisions that could be made related to 4 key life cycle issues, and we do not present the full scenarios matrix for these 4 decisions (e.g., 4 decision points of 2 options $= 2^4 = 16$ total scenarios). Scenarios 1–5 are outlined below and are structured to highlight the impact of making one specific decision between two alternative options for each of the four selected issues:

- Difference between Scenarios 1 and 2—related to gas procurement assumptions about gas flaring
- Difference between Scenarios 2 and 3—related to improvements in bioreactor gas transfer properties
- Difference between Scenarios 3 and 4—related to improved system integration to recover and reuse the residual lipid-extracted biomass
- Difference between Scenarios 4 and 5—related to the introduction of a high-value chemical coproduct (ectoine) and the allocation of impacts between products

Table 16.1 Summary of life cycle case study scenarios

Life cycle issue	Scenario 1	Scenario 2	Scenario 3	Scenario 4	Scenario 5
Methane gas alternate fate	Recovered for use	Flared	Flared	Flared	Flared
Bioconversion technology	Conventional bubble column	Conventional bubble column	Improved bubble column	Improved bubble column	Improved bubble column
Fate of residual biomass	Waste	Waste	Waste	Recycled (anaerobic digestion)	Recycled (anaerobic digestion)
Products	Diesel	Diesel	Diesel	Diesel	Diesel + ectoine

The goal and scope of the life cycle assessment case study were to develop an assessment of the greenhouse gas emissions and ultimate global warming potential of the individual scenarios, represented in terms of equivalent CO_2 emissions (CO_{2eq}). The system boundary considered in each scenario is equivalent and comprises all of the stages discussed above and in Fig. 16.1. For all cases, the primary product being assessed is a renewable diesel fuel product, with an additional coproduct being added in Scenario 5. Life cycle environmental impacts are presented in relationship to our functional unit (1 MJ fuel product) and are described as g CO_{2eq} per MJ of final fuel product. Below, a brief description of the data involved in the life cycle inventory is presented. All inputs of materials and energy and their associated life cycle inventories come from the ecoinvent database unless otherwise noted.

The first life cycle issue assessed is the implications of the alternate fate of the methane-containing gas in the gas procurement life cycle stage. For all scenarios, a consistent gas composition for associated gas was assumed based on observed data from the Bakken oil field in North Dakota (Wocken et al. 2013). In Scenario 1, the associated gas is assumed to have been collected and routed to conventional natural gas infrastructure before the new methane biocatalysis system was introduced. In Scenario 2, however, the assumption was made that methane-containing gas was being flared before the new methane biocatalysis system was introduced. In both cases, we track carbon flows into and out of the system and account for GHG emissions and a complete carbon balance along the entire life cycle. In Scenario 2, however, we have the added benefit of avoiding gas flaring, with release of carbon-containing gas into the atmosphere with no productive use, and the impacts of that assumption are illustrated in the differences in results between Scenarios 1 and 2.

The Bakken oil field gas composition is used to calculate the potential CO_{2eq} emissions savings from avoiding the flaring of this gas, assuming that all carbon-containing gases are converted to CO_2 during flaring. Inputs required for gas extraction from the underground reservoir were quantified in terms of the relative economic value they have in relation to oil, the primary product at these sites. As mentioned previously, prior investigations have determined that associated natural

gas has relatively little economic value compared to the oil recovered at these sites, so a majority of the combined recovery activity should be attributed to the oil product. Based upon a typical natural gas-oil production ratio of 1100 ft^3/barrel (Lutz 2013) and an estimated natural gas price of $3/MMBtu (U.S. EIA 2017), this would equate to roughly $4 worth of associated natural gas per barrel of oil. With oil prices forecasted to remain near or above $50/barrel for the near future (U.S. EIA 2017), a decision was made to estimate an economic allocation for associated gas production at 10% of a standard oil production system, involving inputs of energy to extract oil and gas. This approach may need to be revisited in the future with location-specific data, but in our opinion this is a good first approximation of the appropriate contribution of gas production to an associated gas system in the Bakken reservoir. Pipeline infrastructure was conservatively estimated by assuming that an additional 10 km of pipeline would be required to collect gas and route it from multiple collection wells to a common point for either flaring or collection for future processing. Ecoinvent data related to pipeline network construction was used, assuming a pipeline lifetime of 40 years.

Gas pretreatment was modeled in collaboration with industrial partners, using standard engineering assumptions governing multistage gas compression to determine power requirements for gas compression. These power requirements apply to the methane-containing gas streams required for biocatalysis, as well as a second gas stream required to introduce oxygen into the system, which is a key additive in the biological process. Oxygen was added through the addition of regular air with no purification above typical atmospheric oxygen concentrations (21%) for this study. In separate evaluations, however, the trade-offs between investments in gas purification systems and the resulting decrease in gas compression inputs related to lower overall volumes of input gas were explored in detail, as was discussed above. No impurities were assumed to be removed in the scenarios presented.

The bioconversion process was modeled in collaboration with industrial partners that have extensive experience with conventional bioreactors and several iterations of improved gas transfer reactor systems. In the general descriptions of unit operations described above, it was mentioned that changes in bioreactor gas transfer parameters would reduce the amount of gas compression required to achieve a target level of biological performance from the biocatalysis system. In Scenarios 1 and 2, a conventional bubble reactor is assumed to be used, based on standard engineering estimates of reactor performance. In Scenario 3, an improved bioreactor is modeled, which can increase mass transfer capabilities in the methane aqueous-phase system by a significant amount and consequently reduce power requirements associated with gas compression by well over 50%. The modeled system performance is conservative and has been consistently improving over the course of a related Department of Energy-funded project associated with methane fermentation. Therefore, the observed differences between Scenarios 2 and 3 are solely due to the changes in bioreactor mass transfer performance. Nitrogen, phosphorus, and other nutrients required for growth and maintenance of the biocatalytic organism under study are provided in excess of stoichiometric requirements, in accordance with existing process data. Additional chemicals are added to optimize the properties of

the aqueous phase, and cooling water is also added to maintain a desired operating temperature.

Product separation is assumed to occur via cell disruption and hexane extraction, similar to algal extraction of lipids (ANL 2016). Lipid content of biomass is assumed to be 30%, which is in line with prior expectations of the biocatalytic organism. A significant portion of the aqueous phase is recycled to the bioreactor, with associated water-soluble chemical inputs. In Scenarios 1–3, the leftover non-lipid biomass is presumed to be treated as a waste product, while in Scenario 4, this biomass resource is assumed to be used on-site as a supplemental source of nutrients, heat, and power, which will reduce the external demand of these required inputs. Anaerobic digestion is utilized to process the non-lipid biomass into a methane-containing gas stream, which is combusted in a combined heat and power (CHP) system to generate process heat and power, while releasing carbon-containing waste gases to the atmosphere. A substantial amount of the N and P that was present in chemical constituents of the biomass is also in the aqueous phase, where it can be recycled and returned to the bioreactor system. Key operating assumptions concerning anaerobic digestion are adopted in a similar fashion from the Argonne GREET model treatment of non-lipid algal biomass (ANL 2016). A small amount of solid and liquid waste was left to treat via standard waste treatment modules found in ecoinvent. In this fashion, the differences between Scenarios 3 and 4 illustrate the impacts of careful system integration.

After products are separated, lipid transport and upgrading to the final diesel fuel product are modeled according to the standard hydrodeoxygenation (HDO) process templates found in the GREET model for a typical bio-oil (ANL 2016). This involves a combination of truck and rail transport for average transport within the USA, followed by inputs of process heat, electricity, hydrogen, and other inputs for the HDO process. Diesel fuel has a fairly simple product use and end-of-life phase, because the fuel is simply combusted, with a stoichiometric release of carbon-containing waste gases as described in the GREET model.

In Scenario 5, the life cycle product system is modified by assuming that the biological organism being utilized can also produce ectoine in addition to the primary lipid product. This osmolytic compound plays a significant role in protecting cell components during times of biological stress and is normally produced from culturing halophilic bacteria or other extremophiles (e.g., Van-Thuoc et al. 2010; Lang et al. 2011). Ectoine is a high-value compound with uses in the cosmetics and personal care product industries, with a reported value of $1000 or more (Strong et al. 2016). Methane-utilizing bacteria have been studied as a potential new source of ectoine and other high-value products in a combined biorefinery operation where multiple products are created by a single organism (Strong et al. 2016). The differences between Scenarios 4 and 5 are due to this illustration of potential coproduct allocation impacts.

Many different assumptions were required to develop a biorefinery-based case study, and the ultimate results are likely quite sensitive to these estimates, but Scenario 5 assumptions appear relatively conservative. Economic allocation was chosen as the system to divide environmental impacts between lipids and ectoine for the shared phases of the biorefinery life cycle, because a decision to produce such a

high-value compound would certainly be motivated by economic considerations. Ectoine production was assumed to be 0.5% of total biomass, which is well within production values reported for other ectoine-producing organisms. Ectoine is soluble and is assumed to be extracted from the aqueous phase after cell disruption and prior to hexane extraction of lipids. Further concentration and purification will be required for a commercial product, but we assume a market value for unpurified ectoine of $250/kg at this stage, which is a conservative estimate that leaves plenty of room for investments in purification technology while preserving a healthy profit margin. To determine economic allocation, we need to compare the economic output of ectoine with the economic output of the primary lipid product. A value of $2 per gallon equivalent of lipid ($0.62/kg lipid) was assumed for the lipid, which is in line with Department of Energy production targets of $3 per gallon of final fuel product (Dutta et al. 2015). Even though our combined biorefinery process will produce lipids at 30% of biomass content compared to ectoine at 0.5%, this mass difference is more than made up for by the 400-fold difference in the per kg economic values between ectoine and lipids. As a result of this substantial shift in the economic value outputs of the biorefinery introduced by small amounts of ectoine production, an economic allocation would suggest that 87% of the impacts associated with the shared biorefinery unit operations would be attributable to the ectoine, with only 13% attributable to the lipid product. Impacts associated with lipid processing and fuel combustion are still solely attributed to the lipid product.

16.3.1 Case Study Results

It is apparent that even a relative simple 5-Scenario case study becomes quite complex when evaluating an entire product life cycle, with many unit operations, associated assumptions, and input data. Nevertheless, this case study, based on data from reliable public sources and relevant project partner work, can illustrate important insights related to the key choices that could be made in a methane biocatalysis system. A depiction of the case study results is presented in Fig. 16.2.

Because of the choices made in each of the four key operating conditions presented in Table 16.1, Scenario 1 represents the worst case scenario, where the worst decision is made in each of the four conditions. A gas source is utilized that was not previously being flared, a conventional bubble column reactor is used to introduce gases into the reactor, no attempt to recycle non-lipid biomass is made, and no coproducts are made along with the lipid-based diesel fuel. As a result, overall greenhouse gas emissions for this scenario are roughly eightfold higher than a conventional diesel product (Fig. 16.2, right-hand column) (Elgowainy et al. 2014). This illustrates the important point that despite our use of an innovative bio-based mechanism to take advantage of this methane resource, poor choices made along the product life cycle can still result in a product with poor environmental performance compared to our current standards.

An examination of Scenarios 2, 3, and 4 illustrates the potential improvements that could be made at each key decision point. In Scenario 2, a methane-containing gas

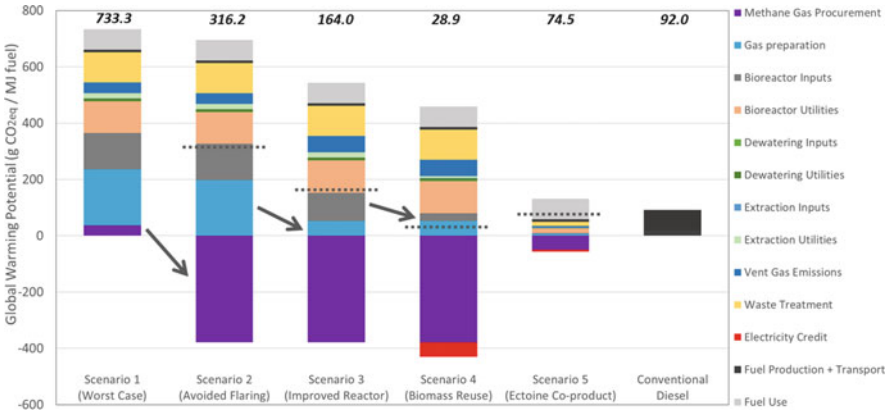

Fig. 16.2 LCA results for methane biocatalysis case study. Five scenarios described above are compared to conventional diesel fuel. Dashed lines present in Scenarios 2, 3, 4, and 5 indicate the overall global warming potential, and numbers above each bar indicate the value of the overall global warming potential in each case. Arrows between Scenarios 1, 2, 3, and 4 indicate the major changes in the results between the scenarios to help interpret the figure

source is chosen that would have been flared if it were not for the addition of this new process, which results in large gains in environmental performance in this system. Environmental impacts associated with gas production at this stage are more than made up for by avoiding the flaring and release of this carbon-containing gas, resulting in a reduction of roughly 400 g CO_{2eq}/MJ diesel fuel. Comparing Scenarios 2 and 3, we can see that a further decrease in emissions of ~150 g CO_{2eq}/MJ diesel fuel (316 down to 164 for Scenarios 2 and 3, respectively) can be realized through the adoption of new bioreactor technology, which increases the mass transfer properties of the reactor and decreases the need for costly and environmentally harmful power inputs to compress all of the methane- and oxygen-containing gases required to maintain reactor performance.

Comparing Scenarios 3 and 4 allows us to illustrate another important process decision, the choice to utilize as much of the biomass as possible for a valuable purpose, instead of treating the non-lipid biomass as a waste product. In Scenario 4, anaerobic digestion was utilized to break down the non-lipid biomass, releasing nutrients which can be recycled and producing process heat to use in the lipid extraction stage. These nutrient and process heat benefits result in the reduction in the gray section of the overall life cycle impact bars in Fig. 16.2 that is highlighted with an arrow between Scenarios 3 and 4. In addition to this improvement, electricity can also be produced from anaerobic digestion, when biogas is produced from the non-lipid portion of the biomass and combusted. This is represented as an additional credit for illustration purposes (red bar in Fig. 16.2, Scenario 4), but in reality this electricity will be used internally to reduce power consumption in other unit operations. As a result of cumulative process decisions changed in Scenarios 2, 3, and 4, overall life cycle impacts of Scenario 4 are projected to be 28.9 g CO_{2eq}/MJ

diesel, which represents a 69% improvement in life cycle emissions compared to conventional diesel.

In Scenario 5, we can see how significantly the environmental impacts of a process may change when a biorefinery approach is taken and new products are isolated from the system. Ectoine is such a high-value chemical product that it distorts the environmental impacts associated with all shared unit operations, but the effect is to reduce impacts of shared processes in a substantial manner (Fig. 16.2, Scenario 5). However, due to the specific combination of process assumptions in Scenario 5, this actually results in an increase in the overall GHG emissions value, compared to Scenario 4. This is because even though the impact of the economic allocation is to reduce impacts associated with the lipid product, it also reduces the positive impacts associated with the avoided gas flaring credit in the gas procurement stage while still requiring the full emissions penalty for fuel production and combustion to be attributed solely to the diesel fuel product. In a sense, Scenario 4 was already a reasonably good outcome, and the added effect of the economic allocation in Scenario 5 was to reduce some of that good outcome and share the majority of the benefit with another coproduct. This does, however, indicate that a biorefinery process will still be worthwhile to evaluate, in an effort to document whether or not specific combinations of ectoine production and market value could be tailored to produce favorable outcomes for the fuel product, as well as the ectoine product (GHG emissions attributed to the ectoine product are not shown in Fig. 16.2). The outcome from Scenario 5 is strictly due to allocation methodology rather than on decisions regarding design choices. Net environmental benefits from the improved biorefinery configuration are still realized to nearly the same extent as shown in Scenario 4. There may also be situations where developing a biorefinery operating scenario can mitigate some of the negative impacts associated with the suboptimal process decisions illustrated in Scenarios 1, 2, and 3. For instance, operating in a biorefinery system with multiple valuable products may allow a company to produce products with clear environmental benefits compared to conventional systems, even if they are unable to utilize flared gas or unable to productively use non-lipid biomass for some technical reason.

In summation, the case study exercise is a good illustration of the potential environmental impacts associated with the full methane biocatalysis life cycle as described in this chapter. It is apparent that a full life cycle view must be taken to realize the potential for environmentally favorable outcomes and to fully understand the impacts that changes in one unit operation may have on upstream or downstream unit operations, resulting in cascading effects on the overall results of the life cycle outcome. By making good decisions across the product life cycle, it does appear possible to produce fuels and chemicals with favorable environmental impacts compared to conventional products, but more study will be required to determine the impacts of specific market opportunities and operating case studies.

Acknowledgments The information, data, or work presented herein was funded in part by the Advanced Research Projects Agency-Energy (ARPA-E), US Department of Energy, under Award Number DE-AR0000438.

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Cracking "Economies of Scale": Biomanufacturing on Methane-Rich Feedstock

17

Anna M. Crumbley and Ramon Gonzalez

Methane is considered a "dream energy package" for chemical production. The large amount of energy stored in methane (CH_4) and the current market trend toward lower CH_4 price make the use of CH_4 as a carbon and energy source for higher-value chemical production desirable. With a substantial stored energy capacity of 47 MJ/kg as determined by the lower heating value (LHV), CH_4 represents a driving force behind only hydrogen (120 MJ/kg) as a high-energy feedstock for chemical production (Boundy et al. 2011). Recent natural gas prices put methane costs around $5/thousand cubic feet in 2016, one of the lowest trends since the early 2000s (Fig. 17.1a; U.S. Energy Information Administration 2017). While CH_4 production volumes are increasing, due in part to transitions in oil and gas recovery techniques (Fig. 17.1b), the distributed and small-scale nature of many sites, together with CH_4's inherent chemical properties including flammability and gaseous nature, complicate its recovery and transportation using conventional technology (Fig. 17.1c; U.S. Energy Information Administration 2016b). Rather, standard protocols at distributed or small-scale sites typically rely on flaring or venting of CH_4 to the atmosphere to remove the gas (U.S. Environmental Protection Agency 1991). Remote CH_4, however, represents a potentially lucrative opportunity given the viable technology to recover and transform CH_4 into a more readily transportable form, such as a liquid or solid value-added product (Haynes and Gonzalez 2014; Conrado and Gonzalez 2014; Clomburg et al. 2017). Unique opportunities for industrial biomanufacturing that are not considered to be feasible for chemical manufacturing via traditional routes may be well-suited to address several challenges

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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_17

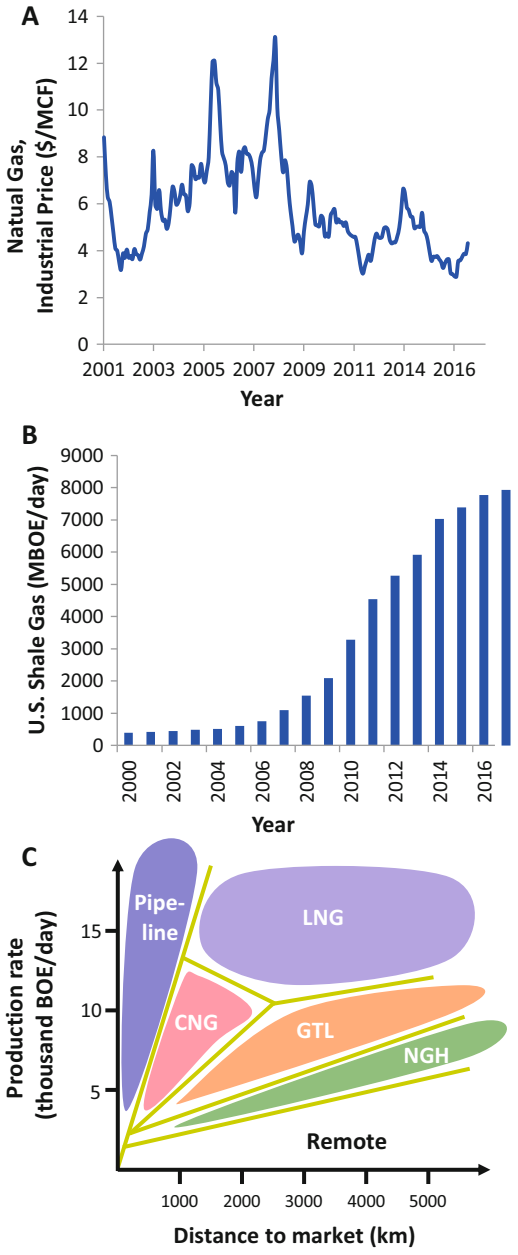

Fig. 17.1 Trends in natural gas and methane recovery. (**a, b**) Natural gas prices for the industrial sector have fallen, and production has risen with the increase in implementation of hydraulic fracturing technology since 2008. MCF, thousand cubic feet. (**c**) Economically feasible strategies for natural gas transport by location. While much of the natural gas produced is recovered, gas produced in remote areas and/or in small quantities is not considered to be economically feasible for recovery through standard operations and is often labeled "remote." Modified from Clomburg et al. (2017) and Khalilpour and Karimi (2010). CNG, compressed natural gas; LNG, liquefied natural gas; GTL, gas to liquids; NGH, natural gas hydrates. Data from references U.S. Energy Information Administration (2016a, 2017)

associated with remote CH_4 recovery, such as utilizing small feedstock volumes, erecting compact facility operations at or near well sites, pursuing biological mechanisms for direct conversion of CH_4 to higher-value fuels and chemicals in liquid or solid form, and considering early separation requirements during process design for efficient scale-up to commercial operation.

17.1 The Economy of Scale Mantra: "Bigger Is Better"

"Bigger is better" has been the status quo for traditional chemical production since its eighteenth-century infancy, when adhering to the economy of scale model meant fewer but larger conversion facilities, feedstock streams, and production volumes with lower production costs. By consolidating and spending less on equipment and dividing those costs across an increase in production volume, a greater profit could be made. According to the economy of scale model, increasing the number of units produced by a single facility proportionally decreases the cost per unit toward an absolute minimum amount. Yet today, the costs associated with constructing these behemoth facilities often reach into the billions of dollars and in some cases require decades of construction labor, limiting the market to a few organizations with the funds and expertise to compete at this scale and long-reaching factory outlooks with facilities often designed for operation over a 10–40-year period (Clomburg et al. 2017). Considering that the financial investments required to construct the modern facilities used for manufacturing are considerable, in some cases on the order of $8–15 billion dollars, large-scale facilities constructed are expected to operate well beyond the time required to recoup expenses (Clomburg et al. 2017). Factoring in modern-day challenges of globalization and raw feedstock diversification, calls for energy efficiency in manufacturing, and a movement toward alternative feedstocks, the "bigger is better" economy of scale model could hinder the ability of the traditional chemical production industry to adapt to change. Potential future challenges in the spheres of environment, geography, politics, and economics include natural changes to local environmental conditions, expansion of the global economy to manufacturing in regions which offer nontraditional resources, political shifts, and emerging economic attractions to recovering and utilizing smaller-scale and waste feedstock resources, such as waste C_1 feedstocks. Therefore, while the economy of scale model has revolutionized modern life over the past few hundred years, the model also potentially leaves traditional chemical manufacturing operations vulnerable to accelerating trend shifts in global manufacturing through slow adoption of change in the chemical manufacturing sphere.

17.2 Industrial Biomanufacturing: Evidence of an Alternative Manufacturing Model

An alternative manufacturing model for chemical production was identified during a case study of corn-based bioethanol plants, the most widely implemented example of commercial industrial biomanufacturing to date. The model, known as the "economy of unit numbers," developed from observations that large numbers of smaller-scale facilities, constructed near the feedstock source, were competing in productivity with single, larger-scale facilities to which large volumes of feedstocks must be ferried from far distances (Fig. 17.2a; Clomburg et al. 2017). "Economies of unit numbers" can be defined as unit production distribution across a series of small-scale processing facilities working in concert to collectively produce a large volume of product. Locating the facilities at or near feedstock sources, such as with the concentration of corn-based bioethanol plants (green marker, Fig. 17.2a) in the grain-rich regions of the United States of America (USA), reduced feedstock storage and transportation costs and incentivized recovery of localized, small-scale resources. Several additional factors likely contributed to the economy of unit number model development in corn-ethanol production, including reduced capital costs due to improvements in technological design and the incorporation of strategic processing practices into successive iterations of bioethanol facility design (Fig. 17.3; Clomburg et al. 2017). The large volume of facilities designed and constructed over the concentrated 30-year period encouraged a modular-like installation development, reducing design and capital expenditure (CapEx) costs and allowing for streamlined manufacturing of major equipment pieces (Boysen 2017).

Remote, distant sources of CH_4 represent a similarly situated feedstock to which economy of unit number principles could be well-suited for methane-based biomanufacturing. Methane's flammable, gaseous nature coupled with its origination in regions distant from transportation facilities or in small quantities makes efficient recovery using presently available methods unfeasible at remote sites. The economy of unit number model represents an alternative manufacturing method which could be initially implemented for the development of industrial biomanufacturing using C_1 feedstocks, such as methane, and later expanded to capitalize on deposits of other small-scale resources.

The compact nature of industrial biomanufacturing lends itself to smaller-scale facilities, a boon when considering the implementation of industrial biomanufacturing processes near smaller-scale feedstock sources. Corn-ethanol production facilities compare favorably with larger refineries when considered on a productivity-per-land area basis (Fig. 17.2c; Clomburg et al. 2017). When hourly output was compared on a per hectare of land basis, as measured via aerial satellite imagery, the Big River United Energy corn-ethanol plant in Dyersville, Iowa, produced output on a barrel of oil equivalent (BOE/h/ha) per hour per hectare of land basis comparable with the ExxonMobil Baytown Refinery in Baytown, Texas. Big River United Energy published a production capacity of 4500 BOE/day on 12 hectares of land in 2016, while ExxonMobil Baytown Refinery published a production capacity of 584,000 BOE/day on 1375 hectares of land during the

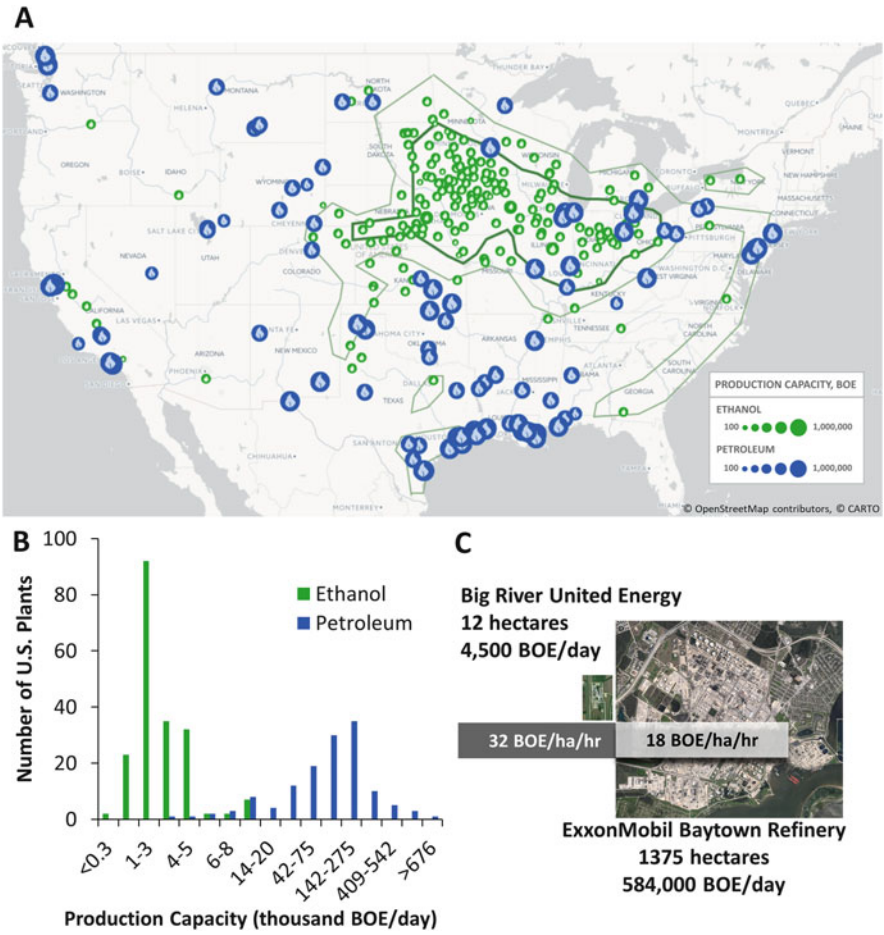

Fig. 17.2 Comparison of petroleum refining to industrial biomanufacturing. (**a**) Geographical distribution of oil refineries (blue/blue flame) and corn-ethanol plants (green/green leaf) in the USA. Corn-ethanol production facilities are concentrated in major corn-growing regions, represented by the interior of the dark green polygonal region. Minor corn-growing regions are represented by the interiors of the multiple lighter green polygonal regions. Icon size correlated with production at each site. Production capacity represented in BOE/day, barrels oil equivalent per day. Adapted from Clomburg et al. (2017). Data obtained from references Brown (2013), CF (2016), Yara (2006), Morgan (2014), O'Brien (2013), Patton (2013), U.S. Energy Information Administration (2016a), U.S. Energy Information Administration (2016c), Investimus Foris (2015), Google Maps (2016a, 2016b), Jessen (2012), Tate and DuPont (2016), Genomatica (2016) and U.S. Department of Agriculture (1994). (Mapping and georeferencing © OpenStreetMap contributors, © CARTO, respectively.) (**b**) Corn-ethanol (light green) and oil refinery (dark blue) frequency distribution in the USA as a function of plant capacity on an equivalent energy basis (thousand BOE/day). Adapted from Clomburg et al. (2017). Data obtained from references Brown (2013), CF (2016), Yara (2006), Morgan (2014), O'Brien (2013), Patton (2013), U.S. Energy Information Administration (2016a), U.S. Energy Information Administration (2016c), Investimus Foris (2015), Google Maps (2016a, 2016b), Jessen (2012), Tate and DuPont (2016), Genomatica (2016) and U.S. Department of Agriculture (1994). (**c**) Satellite images of a corn-ethanol bioconversion facility and a petroleum

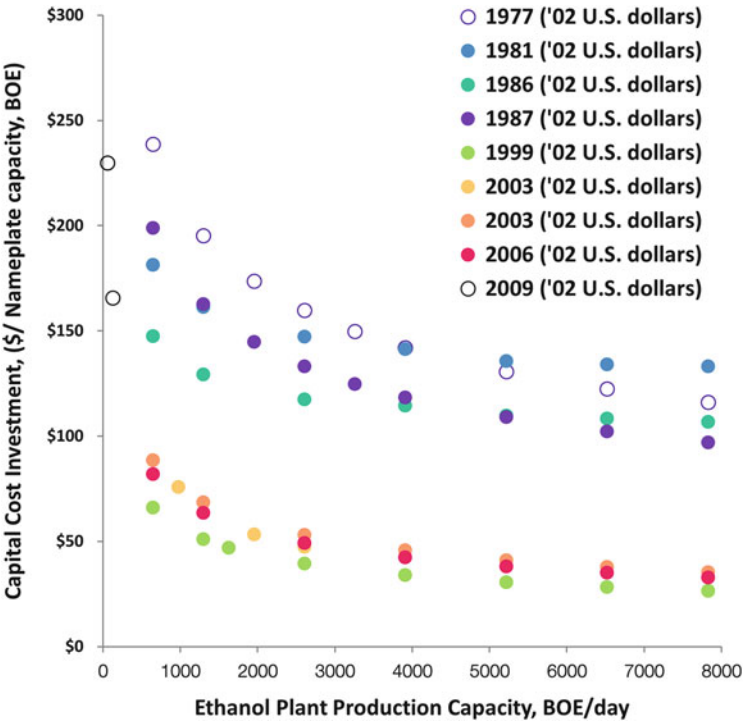

Fig. 17.3 Capital costs for US ethanol plants have been substantially reduced since 1977. Capital costs were shown to be slightly reduced by economy of scale principles within a given study, but non-scale impacts, such as improvements in technological design and the implementation of disruptive technology, were more consequential. Feasibility study markers are represented with hollow centers. All CapEx adjusted to 2002 US dollars for comparison. Adapted from Clomburg et al. (2017) and Hettinga et al. (2009). Data obtained from references Hettinga et al. (2009), David et al. (1978), LeBlanc and Prato (1990), U.S. Department of Agriculture (1986), Whims (2002), Dale and Tyner (2006), Kwiatkowski et al. (2006), Coombe (2009), Cleveland and Kelman (2015), and Ladisch and Svarczkopf (1991)

same time period (Clomburg et al. 2017). When compared using production per land usage as the basis, Big River United Energy produced a rate of 32 BOE/hectare/hour, while ExxonMobil Baytown Refinery produced a rate of 18 BOE/hectare/hour (Clomburg et al. 2017). Considering the remote nature of many CH_4 recovery sites, development of compact industrial biomanufacturing facilities near the CH_4

Fig. 17.2 (continued) refinery emphasize differences in land area coverage. Aerial productivity (BOE/ha/hr) can be calculated for the facilities by considering production capacity and land coverage estimated from Google Map imagery. Adapted from Clomburg et al. (2017) (Imagery © 2016 Google, Map data © 2016 Google)

source would reduce industrial sprawl and facilitate waste gas feedstock recovery and utilization at the point of source.

Analysis of capital expenditure (CapEx), defined as costs associated with start-up equipment and facilities, associated with corn-ethanol plants analyzed for the economy of unit number model indicated a greater trend of decreasing CapEx since 1977 than the traditional economy of scale effect, which projected only a slight decrease in per-unit costs as facility size increased. Standard economy of scale CapEx increases with decreasing production volume and is scaled using a cost ratio such as the 6/10ths rule favored for sizing of standard chemical operations equipment (Turton et al. 2012). Published figures for corn-ethanol facility capital costs from 1977 to 2009 indicate that while CapEx decreased per ethanol unit as the size of the facility increased during any single year, when examined over several decades, the greater trend showed a substantial decrease in CapEx beyond gains made by economy of scale activities alone (Fig. 17.3; Clomburg et al. 2017). Non-scale impacts, such as improvements in technological design, appeare to have occurred through implementation of disruptive technology and feedback learning efforts. One feedback learning model, "learning by doing," incorporates strategies similar to those applied in metabolic engineering and other fields, such as the "design, build, test, learn" model (Clomburg et al. 2017). An "economy of unit number" also suggests a trend toward modular, "off-the-shelf," equipment strategies similar to those trends developing in the pharmaceutical industry, where an entire feedstock conversion process occurs in a single disposable reactor with the goal of reducing interfering external contamination (Warikoo et al. 2012). As the industrial biomanufacturing industry continues to become well-established, a rise in modular-style facility components and manufacturing strategies has the potential to further drop CapEx costs and streamline facility construction.

As biomanufacturing process development advances, biocatalyst technology and bioreactor design will become the primary bottlenecks to utilizing industrial biotechnology as an alternative approach to traditional chemical manufacturing. With a number of tools and strategies, such as CRIPSR/cas9 gene editing, genome sequencing and mapping, and computational modeling of intracellular interactions continuing to become more advanced, research scientists such as those in the fields of metabolic engineering and systems and synthetic biology increasingly have control over the development of chemical pathways and microbial strain production efficiencies (Jakočinas et al. 2015; Dicarlo et al. 2013; Sander and Joung 2014; Warner et al. 2009; Fisher et al. 2014). Three remaining challenges for the bioindustrial sector include increasing biological conversion rates, which are typically lower than chemical conversion rates, overcoming product toxicity to cells, and achieving efficient downstream product separations through increased yields and early strategic focus (Mussatto et al. 2010; Fei et al. 2014; Keasling 1999; Barton et al. 2015; Huang et al. 2014). Specifically when assessing the feasibility of C_1 feedstocks, addressing kinetic challenges for utilization of CH_4, such as through bioreactor design and strategic operation, and identifying more energetically favorable molecule activation strategies remain paramount (Haynes and Gonzalez 2014).

The economy of unit number model represents an alternative manufacturing approach which can facilitate placing smaller-scale manufacturing sites at or near the feedstock source, thereby incentivizing remote feedstock recovery, encouraging market adaptability, and diversifying the industry to include more contributors such as those in a few developing nations which possess substantial volumes of remote CH_4 feedstocks (Clomburg et al. 2017). Potential benefits of applying the economy of unit numbers principle to CH_4-based industrial biomanufacturing include encouraging competition in the areas of design and process engineering research as well as CapEx and operational activities, expanding the commercial industry, and promoting strategic innovation.

17.3 C_1 Feedstocks: Economic and Environmental Impacts of Remote CH_4 Recovery

When considering C_1 waste feedstocks, distributed production facilities become increasingly attractive. Mapping and quantifying small-scale, remote CH_4 feedstocks across the USA identified more than 1900 sites at which methane was flared or vented in 2014, most often in the form of natural gas (Fig. 17.4). While larger-scale methane production sites recover CH_4 via tanker rail car or natural gas pipeline, methane sites defined as "remote" are either located far from processing and transportation facilities or else produce at small volumes and represent economically unfeasible sites for methane recovery using conventional CH_4 recovery processes (Fig. 17.1c). Currently, CH_4 is flared at remote sites, although venting of CH_4 directly to the atmosphere also occurs (U.S. Environmental Protection Agency 1991; Zhang et al. 2015; Elvidge et al. 2009). Flaring of unrecoverable gas is considered the preferred action on an environmental basis, since current estimates place CH_4 as being approximately 25 times more harmful to the atmosphere than an equivalent amount of carbon dioxide (CO_2) over the same period (U.S. Environmental Protection Agency 2017). While the individually small cost of flaring or venting CH_4 at these sites each represents a minor economic and environmental loss, collective analysis of the total non-recovered CH_4 in 2014 equated to nearly 500 billion cubic feet/year (Clomburg et al. 2017). Taking the 2014 year-end average industrial price for natural gas at $5.58/thousand cubic feet, if the entirety of the CH_4 reported to have been flared or released to the atmosphere in 2014 instead had been recovered, it would have represented almost $2.8 billion dollars in economic impact (U.S. Energy Information Administration 2017; Clomburg et al. 2017). In the event the CH_4 were to have been recovered and used as a feedstock for a value-added process such as industrial biomanufacturing, the already substantial economic opportunity would have been greater. In addition to a lost economic impact, the large total volume of methane released in 2014 also had notable environmental impact, potentially equivalent to 835 billion m^3 CO_2, or over 600 times more than estimated US automobile emissions in 2014 (Clomburg et al. 2017; Statistica et al. 2017; U.S. Environmental Protection Agency 2014). Considering global emissions on the order of 4 trillion cubic feet of CH_4 over the same

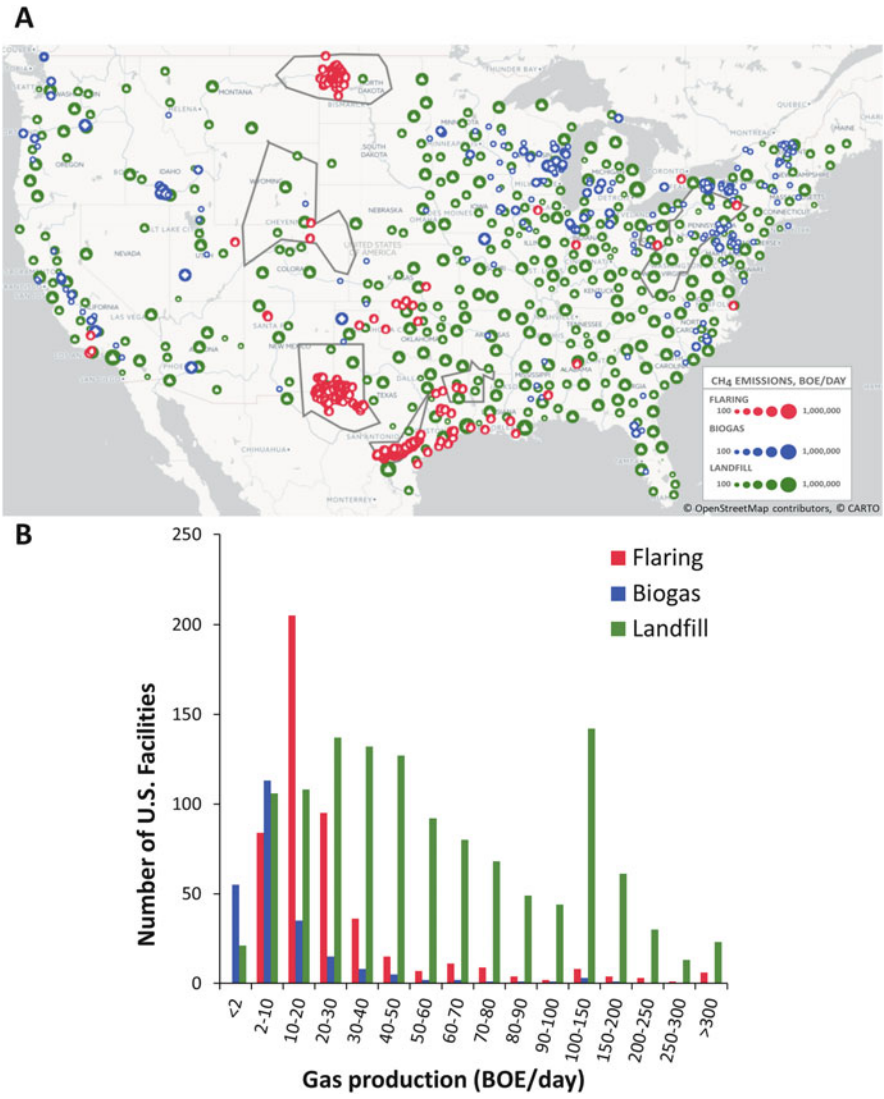

Fig. 17.4 Remote sources of CH_4 are distributed across the USA and represent opportunities for bioconversion-based methane utilization facilities. (**a**) Flaring/venting or capturing of CH_4 at natural gas wells (red), landfill sites (green), and agricultural biogas facilities (blue) in the USA. Natural gas flaring/venting regions are largely correlated with major natural gas production sites, represented by the interiors of the multiple gray polygonal regions. Icon size correlated with production at each site. Production capacity represented in BOE/day, barrel oil equivalent per day. Data obtained from references Zhang et al. (2015), Elvidge (2014), Elvidge et al. (2013), U.S. Environmental Protection Agency (2015), U.S. Energy Information Administration (2016d). (Mapping and georeferencing © OpenStreetMap contributors, © CARTO, respectively.) (**b**) Flaring/venting (red), landfill (green), and agricultural (blue) frequency distribution in the USA as a function of natural gas production rate (BOE/day). Data collected from 490 VIIRS-identified

period, the economic and environmental loss was extensive and also represented enough carbon to satisfy global production of seven essential building block organic chemicals, a list including methanol, ethylene, propylene, butadiene, xylene, benzene, and toluene (Clomburg et al. 2017). Accordingly, developing technology for the capture and conversion of currently underutilized waste CH_4 resources using technology such as C_1-based industrial biomanufacturing represents an attractive opportunity for the industrial biomanufacturing industry to employ economy of unit number principles during the recovery and conversion of remote CH_4 resources.

17.4 Methane Bioconversion: Microbial Incorporation of C_1 Feedstocks

Capture and utilization of small-scale methane resources nationwide through industrial biomanufacturing center on development of robust and efficient microbial organisms which consume methane and other one-carbon feedstocks and produce value-added fuels and chemicals that can be integrated into the consumer market. Methanotrophs are the group of organisms which consume methane and methanol as sole sources of carbon and energy. While historical efforts to use these bacteria industrially were faced with slow growth rates and limited strategies for genetic modification, methanotrophs with faster growth rates have since been identified, such as *Methylomicrobium buryatense* 5GB1, and tools for genetic modification have been developed, including an electroporation protocol, expanding the opportunities for working with methanotrophs and C_1-related systems (Yan et al. 2016; Kaluzhnaya et al. 2001). Additionally, expression of C_1-related enzyme pathways in *E. coli*, an organism commonly used in the biomanufacturing community due to its robust growth, amenability to genetic modification, and extensively developed toolkit, has been reported for consumption and metabolic integration of methanol, although robust heterologous expression of methane monooxygenase (MMO), the primary known aerobic enzyme for methane consumption in bacteria, has yet to be reported (Clomburg et al. 2017; Whitaker et al. 2017; Müller et al. 2015).

Four enzymes are present in aerobic methane oxidation. These include methane monooxygenase (MMO), methanol dehydrogenase (MeDH), formaldehyde dehydrogenase (FalDH), and formate dehydrogenase (FDH). Methane-derived carbon is assimilated at the level of formaldehyde via the ribulose monophosphate (RuMP) cycle or serine cycle, formate via the serine cycle, or CO2 via the Calvin-Benson-Bassham (CBB) cycle, depending on the specific metabolism of the bacterial

Fig. 17.4 (continued) petroleum flaring wells (totaling 16,118.20 BOE/day), 1233 EIA-reporting landfills with capture/flaring capabilities (totaling 88,779.25 BOE/day), and 239 EIA-reporting agricultural sites with biogas generation capabilities (totaling 1597.434 BOE/day) in 2014. Data obtained from references Zhang et al. (2015), Elvidge (2014), Elvidge et al. (2013) and U.S. Environmental Protection Agency (2015)

species, in order to drive biomass production (Kalyuzhnaya et al. 2015; Trotsenko and Murrell 2008). At the formaldehyde branch point, the one-carbon (C_1)-based molecule is assimilated into central metabolism in methanotrophs primarily by either the serine or ribulose monophosphate (RuMP) pathways, depending on the specific characteristics of the bacterial species, in order to drive cell biomass production (Lieberman and Rosenzweig 2004). Figure 17.5 details the pathways for assimilation of one-carbon (C1) products in microbial organisms. A few methanotrophs have the ability to assimilate formaldehyde via both the RuMP and serine pathways (Lieberman and Rosenzweig 2004; Austin and Callaghan 2013).

Methanotrophs are organized into two groups, designated *Gammaproteobacteria* or Group I, which use the RuMP pathway for carbon assimilation, and *Alphaproteobacteria* or Group II, which use the serine cycle (Fei et al. 2014). In addition, thermoacidophilic aerobic methanotrophs (*Verrucomicrobia*) have been identified which can also assimilate carbon through the CBB cycle at the level of CO_2 (Khadem et al. 2011). Furthermore, two novel routes for C_1 incorporation have been reported, including a computationally designed enzymatic pathway and reversal of methanogenesis. The aerobic synthetic pathway, focused on the computationally designed enzyme formolase (FLS), has not been found in nature but was designed to perform an organic chemistry synthesis reaction, coupling formaldehyde to produce dihydroxyacetone (DHA), and was integrated as part of a series of enzymes to construct a formate C_1 incorporation pathway (via formaldehyde) into the cell (Siegel et al. 2015). The reversal of methanogenesis pathway reported the functional reversal of methyl-coenzyme M reductase (Mcr), the final enzyme for methane production during methanogenesis, and was engineered for anaerobic methane consumption in the presence of several electron acceptors using methanogens as hosts (Soo et al. 2016).

Despite having identified several pathways, both natural and synthetic, for C_1 product incorporation into cellular metabolism, methods for activation of methane remain an area of active research since the above strategies continue to be largely limited by energy-inefficient activation of methane. By comparison of lower heating values (LHVs), only 67% of the energy present in CH_4 is still present once the molecule reaches formaldehyde as a result of the substantial energy input (two reducing equivalents) required to activate MMO (Haynes and Gonzalez 2014). One method to address the poor energy efficiency would be to identify or computationally design novel enzymes which activate methane via different chemistry. Similar enzymes, including the monooxygenase class of cytochrome P450s and dioxygenases, have been proposed as models for alternative methane activation enzyme designs (Haynes and Gonzalez 2014; Conrado and Gonzalez 2014). Beyond development of more efficient activation strategies for CH_4, efficient enzymatic and temporal strategies for engineered biological utilization of other C_1 feedstocks also remain active areas of research.

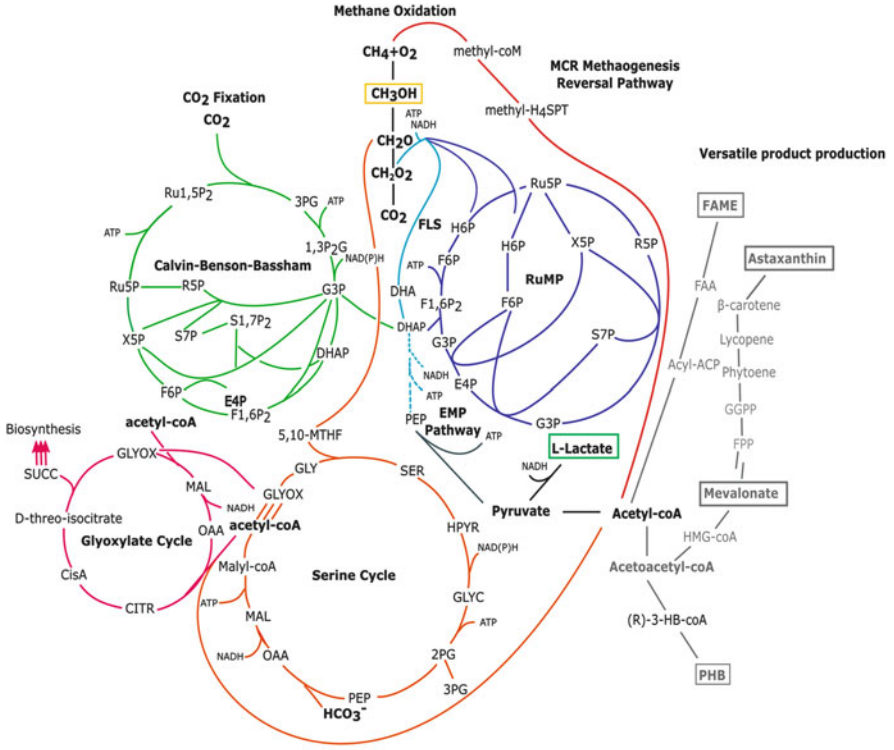

Fig. 17.5 Microbial metabolic pathways for assimilation of C_1 products. The ribulose monophosphate pathway (RuMP; top right, dark blue) incorporates one-carbon (C_1) units via formaldehyde into microbial central carbon metabolism. The serine cycle (bottom left, orange) interacts with the glyoxylate cycle (far left, pink) for biosynthesis and acetyl-coA production. Methanotrophs were recently confirmed to possess the central carbon metabolism Embden-Meyerhof-Parnas (EMP) pathway (Kalyuzhnaya et al. 2015). The Calvin-Benson-Bassham cycle (top left, bright green) for CO_2 fixation, the methanogenesis reversal pathway (MCR) (top right, red), and the computationally designed formolase (FLS) enzyme (middle, light blue) represent other methods for incorporation of C1 products into bacterial carbon metabolism; however, these pathways are either synthetic pathways not found in nature or else not typically found in methanotrophs. Rationally engineered product pathways are shown in gray. Pathways obtained from references Kalyuzhnaya et al. (2015), Siegel et al. (2015), Soo et al. (2016), Sato and Atomi (2001), Liao et al. (2016), Michal (1999) and Flamholz et al. (2013). Abbreviations: Dihydroxyacetone phosphate (DHAP), phosphoenolpyruvate (PEP), ribulose-5-phosphate (Ru5P), ribulose-1,5-phosphate (Ru1,5P2), 3-phosphoglycerate (3PG), 1,3-biphosphoglycerate (1,3P2G), glyceraldehyde 3-phosphate (G3P), fructose 1,6-biphosphate (F1,6P2), fructose 6-phosphate (F6P), xylulose 5-phosphate (X5P), ribose-5-phosphate (R5P), sedoheptulose 7-phosphate (S7P), sedoheptulose 1,7-biphosphate (S1,7P2), erythrose 4-phosphate (E4P), 5,10-methylene tetrahydrofolate (5,10-MTHF), serine (SER), hydroxypyruvate (HPYR), glycerate (GLYC), 2-phosphoglycerate (2PG), 3-phosphoglycerate (3PG), oxaloacetate (OAA), malate (MAL), glyoxylate (GLYOX), glycine (GLY), citrate (CITR), cis-aconite (cisA), succinate (SUCC), acyl-acyl carrier protein (acyl-ACP), free fatty acid (FAA), fatty acid methyl ester (FAME), farnesyl pyrophosphate (FPP), geranylgeranyl pyrophosphate (GGPP), hydroxymethylglutaryl-coA (HMG-coA), (R)-3-hydroxybutyryl-coA ((R)-3-HB-coA), poly(3-hydroxybutyrate) (PHB)

17.5 Continuous Bioprocessing: Shorter Production Times and Fewer Costs?

Batch production has historically dominated commercial biomanufacturing, with motivations for choosing batch production including but not limited to industry-specific quality guidelines, limited or seasonal feedstock availability, and increased operational flexibility (Turton et al. 2012, Warikoo et al. 2012). Industries traditionally employing batch-based biomanufacturing include commercial corn-ethanol and pharmaceutical production, with batch processing representing an attractive solution for chemicals required or safely manufactured only in small amounts, produced from expensive feedstocks, or regulated by stringent controls. However, trade-offs for continuous processing in C_1-based manufacturing in some cases may outweigh traditional batch process benefits. When considering safety, process efficiency, and storage of the gaseous C_1 material methane, downsides of batch processing include substrate and product losses due to spillage, potential for hazardous gas release during material transfer steps, and less stable reactor systems due to frequent start-up and shutdown cycles (Turton et al. 2012). Operating a continuous process while utilizing waste gaseous C_1 feedstocks would facilitate a reduced loss of substrate gas during storage and transport, increased operational safety with feedback regulation designed to prevent fermentation conditions from falling within the CH_4 explosive envelope (5–15% methane in air), and continuous recycling of CH_4 to maximize carbon and energy conversion, as has been demonstrated for hydrogen (H_2) gas culturing systems (Khosravi-Darani et al. 2013). Continuous systems designed with an aim toward steady-state reactor operation also offer two benefits: one, safety records at continuous operation plants are typically higher than those at batch processing facilities and, two, maintaining reactor operation at steady state allows more stringent process control monitoring and completion of fermentation at optimal conditions for peak bioreactor performance (Fig. 17.6; Turton et al. 2012).

As industrial biomanufacturing continues to expand commercially, a transition to continuous processing offers additional opportunities to increase efficiency, such as reducing facility footprint size and streamlining time to product, while also eliminating challenges, such as those associated with intermediate chemical and raw feedstock storage. This would represent a particular benefit for work with explosive gases, such as the C_1 feedstock methane. Additional reported advantages of continuous processes include extended reactor operation at steady state, fewer non-value-added processing steps and an accompanying reduction in required equipment volume capacity, streamlined process flow, shorter feedstock-to-product cycle times, scaling back of required manual labor, and less product spillage compared to batch transfer processes (Warikoo et al. 2012; Maddox and Gutierrez 1996). Accordingly, these opportunities translate to lower CapEx and operational expenditure (OpEx) costs for continuous bioprocessing when compared to batch processing systems.

However, mind-sets of low-risk tolerance, the prevalence of off-the-shelf batch principle-designed equipment, "the legacy effect of depreciated production plants," and regulatory body precedents and stringent oversight requirements are cited as

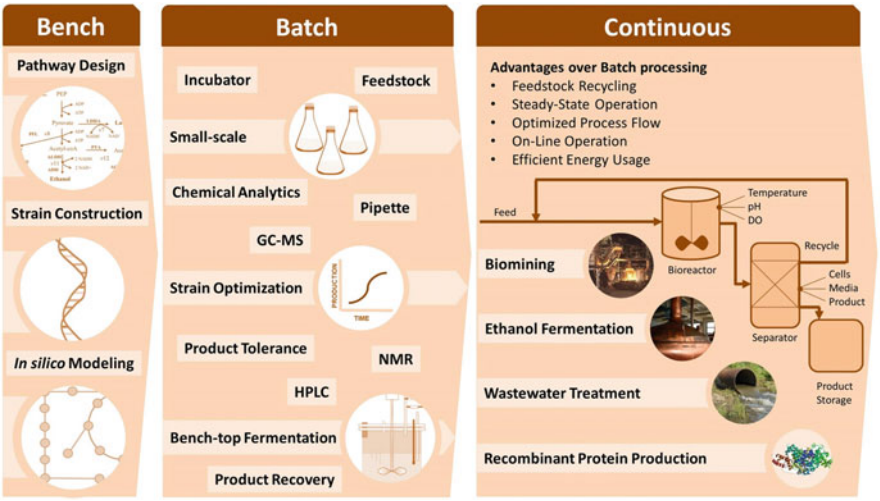

Fig. 17.6 Continuous operation for industrial biomanufacturing. Employing continuous operation concepts can enable feedstock recycling, steady-state operation, and increased process efficiency, and can be developed after initial strain development work and small-scale fermentations are performed

having previously disincentivized a movement toward continuous bioprocessing, in addition to traditional batch process-favoring factors such as seasonal biomass-based feedstocks and slow reaction rates (Turton et al. 2012; Warikoo et al. 2012). Specific concerns to be addressed during the switch to continuous industrial bioprocessing include potential for natural genetic modification of the culture population over time, limited ability to switch products once a process is online, increased equipment sterilization stringency, and, particularly in the case of pharmaceutical processing, a lack of precedent for required regulatory approval (Warikoo et al. 2012; García et al. 2011). Evaluating the impact of these considerations during the early stages of process development is essential to strategic implementation of continuous bioprocessing.

Several commercial bioprocess industries have already or are currently implementing continuous biological manufacturing, including in the areas of water purification, beverage fermentation, and biopharmaceutical production. Other industries have filed continuous bioprocessing patents, although active implementation of the processes has yet to be publicized (García et al. 2011). Specific industries having already invested in continuous bioprocessing include mining and biohydrometallurgy, secondary and tertiary wastewater treatment, ethanol fermentation, and pharmaceutical biochemical production.

The biohydrometallurgy industry has been operating continuous bioleaching reactors commercially since at least 1986, employing microorganisms both to remove sulfides for the extraction of gold from ores and mineral-mixed materials and to recover copper and other minerals from secondary ores such as chalcocite and covellite (Brierley 2008, 1999). Bioleaching can occur either through the removal of

impurities from a solid by microorganisms which solubilize the undesirable metals, or through microbe-driven solubilization of the desired base mineral for downstream recovery (Batty and Rorke 2006). The former technique is typically used for gold recovery, while the latter is employed for obtaining minerals such as copper (Batty and Rorke 2006; Gonzalez et al. 2004). Other base metals recovered through bioleaching include cobalt, nickel, zinc, and molybdenum (Brierley and Brierley 2013). Techniques for downstream copper recovery include a form of electroplating known as electrowinning and solvent extraction (Batty and Rorke 2006). Bacteria employed for bioleaching processes include *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, and *Leptospirillum ferrooxidans*, which oxidize sulfur and sulfides to produce sulfuric acid (H_2SO_4) and in the process generate solutions below a pH of 1.5, increasing the solubility of sulfates and certain metals (Gonzalez et al. 2004; Bosecker 1997). Further degradation of mineral-containing rock by H_2SO_4 produced by the bioleaching bacteria additionally accelerates metal recovery (Gonzalez et al. 2004). Economic studies and commercial optimization analyses have determined that operating multiple bioleaching continuous stirred-tank reactors (CSTRs) in series, as simulates a continuous plug-flow reactor (PFR), results in greater efficiency, with regards to both substrate conversion and operating costs, than comparable industrial batch operations (Brierley 2008; Batty and Rorke 2006; Gonzalez et al. 2004; Brierley and Brierley 2013).

Wastewater treatment processes represent a second long-running application of continuous bioprocessing. Biochemical operations involved in wastewater treatment include processes responsible for removal of soluble organic matter, stabilization of insoluble organic matter, and conversion of soluble inorganic matter in the form of both activated sludge and biological nutrient removal (Grady et al. 2011). Activated sludge purification, a secondary form of wastewater treatment for the removal of suspended and colloidal particles through oxidation, was first developed for wastewater purification in the early twentieth century and is primarily implemented using continuous stirred-tank reactors (CSTRs), although a few batch-based processes have also been developed (Grady et al. 2011; Food and Agriculture Organization of the U. N. 2015). While the early use of multispecies activated sludge was relatively disinterested in the specific functions each bacterial species contributed to the reactor environment, modern efforts have elucidated the roles of many of these species and applied these insights to develop more strategic methods for system management (Yuan and Blackall 2002; Keller et al. 1999). Grady et al. (2011) includes a detailed listing of application strategies and associated reactor types (Grady et al. 2011). Many processes have been developed for secondary and tertiary water treatment, including several recently commercialized Anammox-based nitrogen removal systems (Ma et al. 2016; Sonune and Ghate 2004; van der Star et al. 2007; Singapore National Water Agency 2015). Anammox bacteria-based reactors, which have typically been installed as a tertiary level of wastewater treatment for maintenance of water quality, perform oxidation of ammonium for removal of nitrogen and were developed less than 20 years ago but have already been commercialized and installed to treat nitrogen in over 100 wastewater treatment projects (Food and Agriculture Organization of the U. N. 2015; Ma et al. 2016).

Biological processing has long been a staple of the wastewater treatment community and has contributed substantially to and benefitted from implementing continuous bioprocessing techniques and practices.

Likewise, while the primary model for yeast-based ethanol production globally remains in batch form, a few commercial continuous processes have been implemented. Dominion Breweries (DB), in Auckland, New Zealand, applied continuous fermentation principles to its beer production in 1956, developing a process for producing a classic beer which remains in use today (Campbell 2009; Maule 1986; Cybulski et al. 2011). DB's continuous fermentation process reduced the holding time for the beer product to between 40 and 120 h, substantially less time than the 3 or more week standard for the industry (Cybulski et al. 2011). In 1960, approximately 475,000 gallons of beer were brewed weekly using a continuous fermentation system modeled after the one patented by DB, amounting to more than 260 million 12-oz. cans of beer per year (Maule 1986). While the continuous fermentation process has remained a staple of the DB brewing process for over 60 years, the requirement of strict equipment sterilization standards to prevent contamination has been reported to be a barrier to successful implementation in other breweries (Cybulski et al. 2011).

Brazilian sugarcane ethanol fermentation facilities have also transitioned toward continuous bioprocesses since the installation of Proácool governmental policy in the 1970s aimed at reducing Brazil's dependence on petroleum products for automobiles (Andrietta et al. 2007). Citing expected production increases, reduced nonproduction time, steady-state fermentation conditions, and greater process control, and with governmental policy designed to fund and facilitate installation of "autonomous distilleries" for increased national ethanol production, a shift toward continuous operation occurred in the late 1990s and resulted in between 13–30% of Brazilian ethanol production facilities operating in continuous bioprocess mode, while the remaining 70–87% continued to produce ethanol via a batch process (Andrietta et al. 2007; Lopes et al. 2016; Amorim and Lopes 2005; Amorim 2006). Yet while continuous bioprocesses have historically been marketed as a method to increase ethanol yields, several studies have documented repeated challenges with bacterial contamination, reporting loss of ethanol product and lower yields (Lopes et al. 2016; Godoy et al. 2008; Basso et al. 2011). Complicating findings, these early continuous processes were reported to have stemmed from "low-cost" retrofitting of batch processes, possibly contributing to mixed performance results (Andrietta et al. 2007; Godoy et al. 2008). Regardless, the spread of continuous bioethanol processes in Brazil has been limited, in part due to problems associated with bacterial contamination such as non-yeast substrate consumption and centrifugation impairment (Andrietta et al. 2007; Godoy et al. 2008). Greater efforts to prevent contamination, such as through implementation of strict sterility protocol in combination with design and construction of dedicated continuous systems, would likely be necessary for further implementation of continuous bioethanol facilities in Brazil.

A push to reduce costs, scale, and time to product for pharmaceutical and small-batch chemicals has sparked a recent transition toward disposable, enclosed,

continuous bioprocessing in commercial industries where the batch process mentality has long been a staple of the workplace. With almost 400 approved recombinant protein-based biopharmaceutical products on the market and over 1300 candidates under development in 2015, representing almost 60% of the highest revenue-generating products in oncology care, biopharmaceutical companies have become especially motivated to address the needs of large-scale biological protein production (Sanchez-Garcia et al. 2016). Citing reduced equipment scale and an accompanying reduction in both CapEx and OpEx, Genzyme, Amgen, Glaxo-Klein Smith (GSK), and others have brought on-line or are currently developing small-scale, single-use, continuous pharmaceutical systems (Warikoo et al. 2012; Palmer 2013, 2014). By utilizing disposable, single-use materials, the systems eliminate the need for extensive sterilization protocols. Amgen's $200 million facility began manufacturing monoclonal antibodies in 2014, and GSK's expansion of its Quality Road Singapore facility, is planned to utilize enzyme-based manufacturing for the production of amoxicillin (Palmer 2013; Hernandez 2015; GSK 2015).

Two upstream production methods are primarily utilized in biopharmaceutical continuous bioprocessing; namely, perfusion bioreactors and, for a semicontinuous system, fed-batch bioreactors (Warikoo et al. 2012). Perfusion bioreactors pass fresh media over and around high-density cell material, allowing for containment of cells in the reactor system either by embedding cells in a matrix, filtering cells while removing spent media, or centrifuging spent media to return cells present to the bioreactor (Warikoo et al. 2012). Alternately, fed-batch bioreactors provide one or more components, such as fresh media, to a culture vessel over the course of the run (Warikoo et al. 2012).

Yet while upstream cell growth and product formation have been the focus of early efforts toward continuous bioprocess manufacturing, integration of continuous production with downstream separation technology capable of the speed and volume required for continuous bioprocessing has also been an area of research and development. To address this challenge in its small-scale continuous pharmaceutical process pilot scale operation, Genzyme worked with manufacturers to develop a periodic countercurrent chromatography system which operated in series for product recovery applicable to both more stable monoclonal antibody proteins and less stable recombinant human enzymes (Warikoo et al. 2012). Focusing on product recovery and potential for scale-up during early design discussion is an essential aspect of the biomanufacturing product development process, particularly in light of the commercial transition toward continuous bioprocessing.

Therefore, while current continuous bioprocess systems are largely dominated by wild-type or evolved yeast and bacteria rather than engineered organisms, precedent has been established as to the long-term commercial operation of capable reactor systems, and efforts to scale up separation technology are underway (Warikoo et al. 2012). Favorable economic analyses conducted for bioleaching, and societal pressures for clean water, resulted in early adoption of continuous bioprocessing in a few industries. These pioneers have been followed more recently by the pharmaceutical industry, largely due to the substantial facility space and equipment

reductions afforded by continuous bioprocessing. Looking to CH_4 conversion, continuous bioprocessing offers the opportunity to eliminate extensive CH_4 storage systems, increase gas recycling, encourage online feedback control monitoring, improve reactor stability and product yield, and reduce facility footprint size. The widespread and small-scale nature of remote CH_4 release sites suggests capitalizing on the economic and environmental advantages of remote CH_4 will require innovative solutions with disruptive technology, such as the implementation of novel CH_4 activation routes in collaboration with advancements in industrial biomanufacturing microbial engineering and process design.

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Methanotrophy Goes Commercial: Challenges, Opportunities, and Brief History

18

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18.1 Methane as a Preferred Feedstock

The vast majority of plastics and chemicals used in the manufacturing sector today are derived from fossil fuels. As demand grows for more sustainable, environmentally friendly feedstocks, the focus has shifted to sugars derived from food crops like corn and sugarcane. However, sugars have their own disadvantages: the energy density is much lower than that of hydrocarbons, and their prices are volatile and dependent upon climate conditions. Growing crops also requires vast swaths of land and intensive water use that often compete with production of food for human or livestock use. Second-generation nonfood sugar feedstocks can overcome some of these limitations, but the available technology to extract sugars from cellulosic materials has struggled to compete with more mature processes.

The simplest hydrocarbon, methane, is a sustainable, fungible, and scalable feedstock—and, in the case of biogas obtained from anaerobic digestion, fully renewable. Methane is the main component of natural gas and the end product of anaerobic organic matter decomposition; the world reserves are estimated to be over 139,000 trillion cubic feet (Lee et al. 2016). It is also a very powerful greenhouse gas: in terms of global warming potential over 20 years, the effect of a single ton of methane is equivalent to 86 tons of carbon dioxide (IPCC 2013). Current technologies for utilizing methane focus largely on its use as a fuel, but being a cheap and readily available source of carbon, there is also potential for using it as a building block for making valuable chemicals or to make biomass for nutritional products.

Methane has disadvantages of its own: it is a gas with very low solubility in water, which poses problems with mass transfer, i.e., the fermentation may be limited by

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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_18

the amount of methane that is available to the biocatalysts. In addition, methanotrophs are obligate aerobes. This oxygen requirement raises the concern of generating flammable mixtures. These are challenging but not insurmountable obstacles to commercial-scale fermentation; and the rewards are well worth the effort.

Efforts to produce methane-derived biomass for nutritional purposes at commercial scale are already underway. Food security is a serious concern in the near future—protein production as we know it will struggle to keep up with the increasing demand of an expanding global population. Current agricultural methods require extensive use of land for growing feed for livestock; only a fraction of available land is used to grow food for direct human consumption. The Western world consumes as much as 176 pounds of meat per capita per year ("Livestock, Environment and Development (LEAD) initiative," 2012). With rising standards of living, it is expected that demand for protein in the rest of the world will eventually match that level. In that case, the land required using current methods of agriculture would be two-thirds more than what is presently used—clearly an impossibility (Carbon Trust 2016). Unsustainable demand also plagues aquaculture: preferred seafood like salmon and shrimp need protein in their diet, which currently comes in the form of processed smaller fish. This creates an undue pressure on the ocean population and has sent prices of fishmeal skyrocketing. The importance of meeting this demand in a way that is orthogonal from the human food chain, and with minimal environmental impact, cannot be overstated.

18.2 Methanotroph Biocatalyst Development

Methanotrophy, the mechanism by which methane is assimilated into biomass, is a process carried out by specialized bacteria called methanotrophs, which are obligate aerobes that can only use a few C1 compounds (typically methane or methanol) as carbon and energy sources. Despite their restrictive diets, these organisms are widely distributed in nature and play a key role in the carbon cycle. Methanotrophs have been isolated from many different environments, including freshwater and marine, soils, sediments, acidic peatlands, rice paddies, alkaline soda lakes, and hot springs and even highly acidic thermophilic environments (Handbook of Hydrocarbon and Lipid Microbiology, 2000). Their ability to synthesize higher molecular weight organic compounds has generated considerable interest in their utilization in industrial processes for production of feed supplements and bio-based chemicals.

In theory, the rich variety of environments that support methanotrophy suggests that organisms can be selected to favor a particular fermentation process (e.g., high/low pH, high/low temperatures, high salinity, etc.) that is adequate for the product of interest. However, the majority of these bacteria are either difficult to work with under laboratory conditions or have metabolic requirements that make them unsuitable for industrial purposes. The noncanonical nature of C1 metabolism adds a degree of difficulty to the metabolic engineering of these organisms (Kalyuzhnaya et al. 2015). Even fundamental matters such as the genetic basis of obligate methanotrophy have not been satisfactorily answered (Trotsenko and Murrell

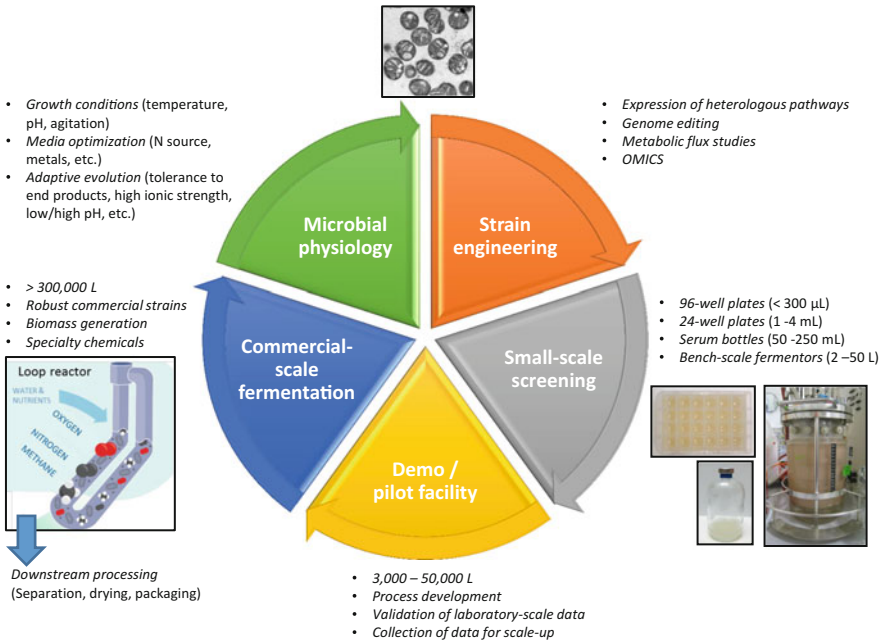

Fig. 18.1 Technological R&D for commercialization of methanotrophic processes

2008; Wood et al. 2004). Other key issues like the link between carbon and nitrogen metabolism or sulfur assimilation also remain unclear.

Despite the biological and engineering hurdles, methanotrophs offer a path to commercialization, especially with production of single-cell protein. But while wild-type, nongenetically modified methanotrophs are suitable for biomass accumulation, generation of nutritionally enhanced strains or production of bulk or specialty chemicals requires careful pathway design, organism optimization, and custom process development. The journey of a particular strain from laboratory to commercial scale (Fig. 18.1) usually starts with gathering fundamental understanding of the physiology of the microorganism. Only a handful of methanotrophs have been studied extensively enough to be candidates for platform organisms. In particular, *Methylococcus capsulatus* Bath is one of the best understood and the only one so far that has been proven at the commercial scale. This type X methanotroph has a relatively fast growth rate at neutral pH in medium with simple salts. Its genome sequence is in the public domain, and the available genetic toolkit allows for the expression of genes from episomal, inducible vectors as well as chromosomal integrations and deletions. *M. capsulatus* Bath has been used as a platform to produce lactic acid, a building block to manufacture polylactic acid, and advanced biofuel isobutanol, among other products. Members of the *Methylomicrobium* genus have also been presented as industrially relevant methanotrophs, with available genome sequences (Vuilleumier et al. 2012), genetic tools (Ojala et al. 2011) and,

in some cases, metabolic models (Torre et al. 2015). Production of lactic acid has been recently demonstrated in *M. buryatense* (Henard et al. 2016).

After strain engineering, candidates for production strains are first assessed in a variety of small-scale formats to measure growth, viability, target product titers, and productivity. Plate formats can be readily adapted for high-throughput screening of desired characteristics, but relatively poor mass transfer and lack of control over key process parameters such as pH are constraints to proper evaluation of strains. Bench-scale fermentors, which allow more efficient mass transfer and control over pH and media composition and off-gas analysis, are paramount to process development. In addition to batch or fed-batch mode, fermentors can be used in continuous mode in adaptive evolution projects, which are often necessary to develop hosts tolerant to end products and/or resistant to specific conditions faced during industrial-scale fermentation (e.g., high salinity, high/low pH, etc.). Strains and fermentation data optimized at this stage are then validated and fine-tuned in pilot plants and demo facilities, a critical step to collect enough information about the process to scale up to commercial level, including safety and regulatory issues. These demo facilities can also serve to manufacture and introduce the final product to the market before committing to building expensive, full-scale plants.

18.3 Past to Present: Brief History of Commercial Methane Fermentation

The idea of using methane fermentation to produce single-cell protein was first explored by Lars Jørgensen as part of his doctorate studies in 1986. This pioneering work was the foundation for a company called Dansk BioProtein, which intended to commercialize this process. The potential of this technology was quickly recognized by Nycomed, a Norwegian pharmaceutical company involved in the discovery of radiocontrast agents, and Statoil, Norway's state-run oil and gas company. Both became early investors in Dansk BioProtein, giving birth to NorFerm, the first company to ever attempt commercial-scale gas fermentation.

Commercial-scale fermentation using a hydrophobic gas as substrate is not without challenges. For example, gases cannot be used by microbes directly; they must be first dissolved in the fermentation broth before they can be taken up. This is a generic problem to all fermentations that use gases, including oxygen, but becomes more acute with large concentration of microorganisms, high demand for gaseous substrates (as is the case with methanotrophs), or high fermentation temperatures that tend to lower the solubility of the gases. Thus, improving mass transfer is paramount for commercially relevant processes. Conventional stirred-tank fermentors generate turbulence in the liquid, which causes the gas, typically injected at the bottom, to dissipate in the form of small fine bubbles. However, the vigorous mixing necessary to achieve this effect requires excessive energy that results in significant heating of the fermentation liquid. This problem can be solved with coolant systems, but the cost would render this type of reactor uneconomical for low-value products such as microbial cells. Furthermore, small gas bubbles have a

tendency to coalesce into larger bubbles as they travel upward, which diminishes the efficiency of mass transfer.

NorFerm tackled this challenge by designing a reactor specifically conceived to maximize mass transfer known as a "U-loop" fermentor (Eriksen et al. 2009). The U-loop fermentor consists of a U-shaped component with a vertical downflow part, a vertical upflow part, a horizontal component that connects the downflow with the upflow part, and a top compartment for gas exchange. The U-shaped part has means to create liquid circulation in the fermentor and one or more gas injection points for the introduction and dispersion of the gas(es) into the fermentation broth. The hydrostatic pressure can be controlled differently in different zones of the fermentor by separate devices, which combined with appropriately spaced static mixers in the upflow part counteract the coalescence of the bubbles. This configuration greatly improves mass transfer, allowing optimal fermentation processes with the highest possible yields in the shortest amount of time. A patent for the U-loop fermentor was awarded in 1989, which gave way to the construction of a 20 m^3 pilot plant in Odense, Denmark.

NorFerm then started making a single-cell protein product using the well-characterized methanotroph *Methylococcus capsulatus* Bath. This organism was chosen because it provided key features, such as inexpensive media requirements and the ability to thrive at temperatures as high as 45 °C, which minimizes contamination and reduces the need for cooling systems. Moreover, its biomass composition has a quality protein and fatty acid profile that makes it ideal for animal feed. Methanotroph-based single-cell protein was introduced to the market under the brand name BioProtein. In 1992, after extensive testing in different livestock, including salmon, trout, pig, and calf, BioProtein was approved for use in animal feed in the European Union.

The Odense plant operated until 1994, when construction of a demo plant began at Statoil's Tjeldbergodden industrial complex in Norway. This new facility operated between 1998 and 2006, making great contributions to the validation of large-scale gas fermentation by perfecting the U-loop design. Many technical challenges were solved during this period, such as reducing the accumulation of carbon dioxide in the broth, which would otherwise slow down growth of cultures, compromising productivity. Also, removal of undesired organic acids released by the methanotrophs was handled by heterotrophic organisms (*Alcaligenes acidovorans*, *Bacillus brevis*, *Bacillus firmus*) naturally present in low numbers among the bacterial population in the broth, which was not maintained in fully sterile conditions. By early 2002, the process met the specifications in terms of kilotons of single-cell protein per fermentor per year. Despite the technical success, a combination of low prices of protein and increasing prices of natural gas in Norway created poor economic incentives for methane-based fermentation. As a result, the Tjeldbergodden facility was decommissioned.

In 2006, a new company named BioProtein AS was formed and received a license to use NorFerm's technology. BioProtein AS engaged in more research about the safety of its single-cell protein product, and in 2011 the approval for use in the European Union was reconfirmed. The intellectual property from Statoil, still a

nominal part of the company, was transferred to BioProtein AS. In 2014, BioProtein AS merged with Calysta Inc., based in Menlo Park, California, with the purpose of bringing methanotroph-based single-cell protein back to the market. In 2016, Cargill and Calysta became partners to build a production plant for the commercialization of methane-derived nutritional products, seeking to seize the opportunity presented by historically low natural gas prices in the United States and a growing demand for reliable, sustainable protein sources.

Methane fermentation is becoming a viable commercial alternative to more traditional liquid-based feedstocks. In addition to the already established technology for nutritional products, there are currently several ventures working to scale up the production of bio-based chemicals such as lactic acid, polyhydroxybutyrate, isobutanol, and farnesene. With further development of methane-ready biocatalysts, along with advances in gas-based fermentation, commercial methanotrophy is poised to become an important contributor to the chemical space.

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Commercializing Innovative Technology

19

Bryan Yeh

19.1 Introduction

Perhaps the most critical step an organization needs to do to prepare itself for commercialization is to establish and implement a disciplined process for guiding it from the idea generation stage through commercialization. Numerous well-established processes such as front-end loading (FEL), front-end engineering design (FEED), and front-end planning (FEP) are used and are all based on the stage-gate process. For this chapter, we will refer to the process as stage gate. Stage gate is a method for managing process/product development and is comprised of stages that consist of prescribed activities for evaluating the process/product opportunity and furthering its commercial and technical development. At the end of each stage is a gate, which is a decision point for committing greater resources and moving the project to the next stage. Checkpoints for specific issues, called milestones, may occur within a stage. Milestone issues must be resolved before additional resources are committed to complete the stage. This chapter provides *guidelines* for the stage-gate process; each process/product development case may vary, and the stage-gate committee may need to alter the process while keeping in the spirit of these guidelines. A graphical depiction of the stage-gate process is shown in Fig. 19.1.

The objectives of stage gate are to:

- Provide systematic approach for increasing project definition.
- Lower risk to positively impact total investment costs and return on investment.
- Make process, design, and business case changes early to keep costs low.
- Allocate resources commensurate with increasing project confidence.

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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_19

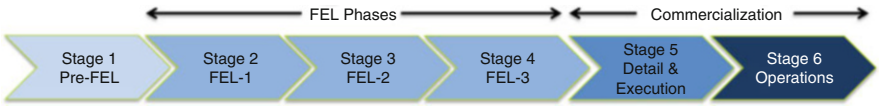


Fig. 19.1 Stage-gate process

Table 19.1 Stage 1 vetting criteria

Commercial	Technical
Identify target market size and growth	First pass at technical concept
Level of interest and need	Identify technical barriers and solutions
Idea on how to go to market	First pass at technology issues
Identify key competitors	First pass at energy and mass balances
Identify partnership opportunities	First pass at program costs

19.2 Stage Overview

There are typically six stages a process/product idea passes through on its way to commercialization. The process is typically managed by a stage-gate committee that comprises individuals from the commercial, technical, legal, financial, and regulatory perspective. The distinct purpose and procedures of each stage for furthering the idea's technical and commercial development are described below.

19.2.1 Stage 1: Idea Vetting

Once an idea is submitted, it should go through a vetting process that determines whether or not the idea becomes a concept that should be pursued. This process is the first stage and comprises both business and technical questions that should be answered. Table 19.1 shows examples of information that should be collected for Stage 1.

Ideas may come from either the "commercial" or the "technical" side of the business; however, regardless of which side of the business an idea comes from, it is necessary to use what is often referred to as a "techno-economic" approach to determining an idea's strength. For example, while vetting an idea, it may be determined that the technical merits are strong, but no market exists for the product. Alternatively, one could have a scenario where the preliminary market analysis shows an unmet need, but the technical challenges for producing the product are significant. Both of these scenarios would probably lead to a negative outcome.

The key to a successful Stage 1 is to use minimal resources to yield the information necessary to make a go/no-go decision. Some key questions that one should be able to answer are:

- Does the market size and corresponding market growth support an entry of another player and the cost of developing the idea?
- How significant are the technical issues, and does the organization have the resources necessary or is able to acquire the resources necessary to resolve the technical issues?
- How much value can be generated by this opportunity?
- Would this idea result in a product with higher quality? Would it result in a product with lower cost?

Throughout the stage-gate process, these questions will be asked over and over again, but as the team acquires more confidence in the project, it will be able to answer these with greater fidelity.

19.2.2 Stage 2: Concept Definition

Once an idea passes the first stage, more time and resources are needed to fully define the concept and to start to test it for fatal flaws. Since the resource needs are greater, it is critical that the idea is fully defined such that individuals from different groups can fully understand the idea. Fatal flaws, such as severe technical challenges or lack of a clear market opportunity may be such that they cannot be overcome. Lastly, it is important to determine the ideas strategic fit into the business and whether or not the organization can appropriately staff the effort. Key focus areas for Stage 2 are shown in Table 19.2.

At this point, it is appropriate to perform some benchtop work to determine the idea's technical feasibility. In the case of engineering microorganisms, the organization may want to deploy limited resources toward engineering a pathway to demonstrate that the production of a given compound is feasible. When doing this work, it is also necessary to develop appropriate analytical methods to properly quantify the work's success. Block flow diagrams are helpful for determining process options and evaluating what the potential capital and operating costs could be. During this step, it is also important to determine the validity of assumptions that are used. For example,

Table 19.2 Stage 2 vetting criteria

Commercial	Technical
Market strategy development	Analytical methods
Identify potential customers	Develop process options
Understand barriers to entry	Benchtop work to determine feasibility
Pro forma economics	Define product attributes

Table 19.3 Stage 3 vetting criteria

Commercial	Technical
Customer samples and feedback	Lab-scale production process
Potential offtake agreements	Capital/operating cost estimates
Refine techno-economic model	Address technical hurdles to achieve necessary economics
Coproduct disposition	Product specifications
Project finance	Technology review

if your fermentation process requires a certain amount of air, determine the size of blowers that are commercially available to determine how many you need.

While the work is proceeding on the technical side, resources should be made available to further develop the organization's understanding of the commercial opportunity. Market reports are useful for framing and enhancing domain knowledge on the landscape. A SWOT (strength/weakness/opportunity/threat) analysis can further help define the significance of the idea. Lastly, a techno-economic model should be made to refine the assumptions of the projects. It should show the team the effect of criteria such as raw material costs, productivity, yield, and selling price has on the net present value (NPV) and internal rate of return (IRR) that the project would have.

19.2.3 Stage 3: Concept Analysis

At Stage 3, there will be additional time and resources spent to identify the issues that must be resolved for development and commercialization to succeed. At this stage, significant effort is placed to define the process and facility that will be used for the commercial plant as well as strengthening information regarding the business case. Table 19.3 highlights some of the relevant considerations.

It is expected that at this time, a lab will be fortified with the equipment and infrastructure necessary to do and perform a lab-scale production process. This will include everything necessary for strain engineering, screening, strain evaluation, process development, and process engineering. Analytical methods should be fully developed to accurately and expeditiously obtain experimental data. Due to the large quantity of information being handled, having tools such as a lab information management system (LIMS) is helpful in accelerating information processing and knowledge generation.

In this stage, engineering firms are often used to help refine and organize information necessary to both define the process and to identify and address concerns regarding the technology. These companies often use the front-end loading (FEL) methodology to produce a package of reports that help depict the commercial process given the knowledge that is available at that time. These reports can include areas such as cost analysis, process modeling using a simulator like ASPEN, safety considerations, technical analysis, and drawings such as a site layout or process and

instrumentation diagrams (P&ID). With this work, it will be possible to provide the basis of a pilot plant that can be used to further demonstrate and define the process.

At this point in time, there should be preliminary discussions with potential customers of the product. In addition to validating any pricing considerations, obtaining product specifications is helpful in defining additional processing that may be required. In biological processes, it is often difficult to determine all of the products that may be produced in the process. As such, obtaining a product specification from a potential client can give one insight on what the customer considers to be critical keeping in mind that the biological process is probably different from the process used for their current source. When the process is different, it is possible that coproducts from the new process have not been contemplated by the client and not specified on the specification. If possible, a sample of the proposed product should be evaluated by the client so that feedback can be obtained and the learning addressed in the process.

Disposition of coproducts should be addressed at this time. Samples of coproducts should be sent to a certified laboratory to determine its relevance and value. In addition, the fermentation broth should be screened to quantify any other metabolites that may affect product recovery as well as either add or detract value. When the value is better understood, techno-economic models should be run to determine what changes there are to the feasibility of the project.

19.2.4 Stage 4: Concept Detail

When the project reaches this stage, resources are deployed to flush out any remaining concerns, both commercial and technical. The objective is to freeze the process, to continue work on the strains, and to lock down supply and offtake agreements. Table 19.4 lists some of the activities for this stage.

A pilot plant that depicts the process as defined by Stage 3 should be built and operated to yield data necessary for the design of the commercial facility. In most cases, the team will find that changes will need to be made to further improve the process, so the pilot plant should be built to accommodate frequent changes. Safety and operability are significant considerations at this time, so work should also evaluate design changes necessary to safely operate a large facility with minimal staffing.

Once the pilot plant yields information that is repeatable and determined to be sufficient for final design, an engineering company can be brought in to start the

Table 19.4 Stage 4 vetting criteria

Commercial	Technical
Customer relationships	Pilot plant
Customer feedback	Production process frozen
Finalize capital and operating costs	Basic engineering package
Project finance	Ensure technology rights

basic engineering package. This package will complete the process design work and specify equipment for the process. An environmental report is usually prepared to facilitate the environmental permit process. There will also be a report that specifies the infrastructure necessary to run a commercial facility. This information will be important for performing site selection. Technology rights should be addressed at this time, and the legal team should be brought in to re-confirm and re-validate freedom to operate.

Site selection is done during this phase and needs to consider the infrastructure requirements, as well as access to raw materials, shipping to customers, availability of labor, ease of obtaining building and operating permits, and suitability of site for construction. In some cases, it may be favorable to purchase an existing, developed site and do minor modifications. In other cases, having a greenfield gives more versatility in terms of getting the site that one needs.

Customers should be identified at this time and agreements in place to set the terms of the business relationship. The terms typically include how the product may be shipped, which will help set the criteria for site selection as well as any infrastructure needed. Product testing that was started in Stage 3 should be continued in this stage to finalize the product specification.

Finally, the capital and operating costs of the commercial plant should be frozen toward the end of this phase to help put together project financing for the construction of the commercial plant.

19.2.5 Stage 5: Execution

During the execution stage of the project, any issues with the environmental permit should be resolved, and final design engineering of the project should be performed. The final design usually considers work such as ground preparation, foundations and building design, equipment layout, site infrastructure, utilities and tie-ins, offices, workshops, and locker rooms. Once this is completed, the next steps are to bid different workout and manage construction of the project.

While the project is under construction, hiring of staff is necessary, from plant employees, to maintenance workers, to utility personnel to office and accounting staff. Training will be necessary for plant employees. If plant operations are new to the organization, additional training for management in areas such as human resources and process engineering will be necessary.

If any strain engineering is continuing, it will be limited to developing strains in accordance to the process that has been chosen for the commercial facility.

Once the facility is substantially complete, the systematic checkout of the facility is necessary prior to start-up. Typically parts of the facility are checked out according to a preestablished punch list that guides the team in terms of what needs to be checked out and the criteria for completing the punch list. Dry runs are made to check operability and to make sure that the facility operates in an integrated way.

As the facility is being commissioned, the team should consider how to handle any off-specification product that may be made. In most cases, such products are

stored in barrels or other containers for reprocessing. Commissioning usually requires additional staff to cover evening shifts and weekend work.

19.2.6 Stage 6: Operations

This is the stage that every team wants to get to. Once the plant is commissioned, it is turned over to the team that will have responsibility for running the facility as an ongoing business. It is quite likely that as the plant operates, the team will find areas of improvement to further optimize the process. If the team hasn't done so already, establishing a balanced scorecard as a means of tracking the operations health is a good way to foster continuous improvement.

19.3 Evaluation Criteria

The evaluation criteria are usually organization specific; however, the methodology below can provide a guideline for how one may approach evaluating the project at each gate.

19.3.1 Project Score Calculation

Step 1—Calculate NPV.
- 10 years of margin from commercialization date.
- No terminal value.
- Include manufacturing capital investment.
- Include non-reusable research capital.
- Use a 25% cost of capital.

Step 2—Calculate developmental manpower costs.
- Estimate person-years.
- Value at $200 K/person-year.

Step 3—Calculate project risk score using the guidelines below.
Step 4—Calculate project risk score = risk factor* NPV/manpower costs.
A grid showing a scoring methodology for determining project risk is presented in Table 19.5.

Technical Risk
Project Development Challenge

This rating considers the risk of new product development by company:

10—A target product that is completely defined; no uncertainty in product form, purity, structure, formulation, etc.

1—A target product that is essentially unknown; for example, a customer wants a product with a certain functional effect, but the product is otherwise undefined.

Table 19.5 Project risk scores (detailed explanation of each score follows table)

Technical	Market
Product development challenge	*Customer development challenge*
10—Existing product	10—Existing product
7—Slight modification	7—Minor product change
3—Major modification	3—Major product change
1—New molecule	1—New product
Process difficulty	*Competition*
10—Existing Process	10—None
7—New process but well-known internal technology	7—Inferior indirect
3—Known technology, but external	3—Inferior direct
1—New to the world technology	1—Strong direct
Proprietary position	*Market penetration to achieve NPV*
10—Unassailable	10—<10%
7—Solid application patent or major trade secret	7—30%
3—Process patent or weak trade secret	3—70%
1—No proprietary position	1—>90%

2–9—Various levels of uncertainty about the active components of blends, possible unknown impurities, and other factors.

Process Difficulty

This rating considers the risk inherent in the contemplated process development activities:

10—A totally commercialized company process produces the target product.

1—The target product will be made by a technology that is known to be commercially practiced.

2–9—New variations of internally practiced technology; first company implementation of technology practiced elsewhere.

Proprietary Position

This rating measures the strength of our proprietary position:

10—A virtually impregnable position, such as a broad enforceable composition of matter patent or sole possession of a critical raw material.

1—No known barrier to competitive entry.

2–9—The protection afforded by process and application patents or trade secrets.

Market Risk

Customer Development Challenge

This rating captures the inherent risk in our customer's development project. It assumes that we will be selling to a customer who must incorporate our product into an end product delivered to a consumer:

10—Our product is a direct drop-in to an already commercially successful product; no reformulation, process changes, or perception changes in end consumer.

1—Our product will be incorporated into a totally new product positioned to offer a new benefit to the consumer; we may be successful in our development project, but commercial success may not occur due to failure of our customer's launch.

2–9—Intermediate values represent various levels of development by our customer or the reduction of risk if many customers are launching products concurrently.

Competition

This rating captures the strength of current or anticipated competitive offerings. The key points are the similarity of the offerings and the manufacturing cost position of the competitive supplier:

10—No foreseeable method that could compete economically with the selling price of our product.

1—An identical product offering is/will be available at a lower manufacturing cost.

2–9—Less threatening cost positions; indirect competition that can deliver a similar product, but uses a different manufacturing route that may have intangible barriers to entry.

Market Penetration

This rating adjusts the project risk to account for market penetration assumed in the NPV calculation. Only the truly addressable market should be considered. High penetration assumptions are inherently risky because of resistance of the marketplace to accept a single solution to a problem.

19.4 Resources

Two types of teams are utilized throughout the stage-gate process: a stage-gate committee and project teams. In addition to the team descriptions, specific role assignments that are stage-gate best practices are summarized below.

19.4.1 Stage-Gate Committee

The stage-gate committee is a stable, standing team with three primary responsibilities: make decisions at the gates, guide product development between gates, and set product development priorities. Keeping committee membership stable across projects yields consistent decision-making.

It is recommended the stage-gate committee be made up of members leading the following functional areas:

1. Business leader
2. Finance
3. Technical research and development
4. Sales and marketing
5. Commercial product development
6. Manufacturing operations
7. Regulatory affairs

19.4.2 Project Teams

The stage-gate committee assigns project teams when an idea is advanced to stage 2. The project teams are project specific and, in general, consist of a technical leader, a commercial leader, and project support. It is recommended that the committee strives for continuity in assigning project team membership as projects move through the stages.

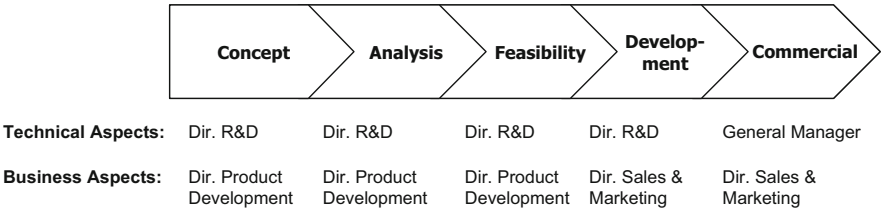
The technical leader is typically from the research and development group but may also be from another functional area such as engineering. Primary responsibilities include research and development activities (e.g., analytical chemistry, separations, synthetic chemistry, fermentation, engineering, product formulation, and intellectual property).

The commercial leader is typically a business analyst, business development manager, marketing manager, or product manager. Primarily responsibilities include business development activities (e.g., market and competitor assessments, financial evaluations, regulatory, safety and efficacy, and customer development).

The project leaders will work together to construct detailed timelines, milestones, and budgets for the project. The milestones should be chosen such that meeting milestone timing is a sound indication that the project is on track. Project leaders are responsible for notifying the stage-gate committee in advance if a milestone or budget is going to be missed. Project leaders cannot exceed approved budgets without approval from the committee. Missing a milestone is not necessarily a failure of the leaders or the team because technical development projects have some risk of failure inherent in them. However, not informing the stage-gate committee promptly when a risk of milestone failure exists is considered a serious failure of the project leaders.

Other aspects that will be coordinated by the project leaders include:

- Development and delivery of gate presentations.
- Maintain and file required documentation.
- Communicate with project team and stage-gate committee.
- Respond to ad hoc questions from the stage-gate committee.



19.5 Meetings

Two types of formal meetings occur during the stage-gate process: committee meetings and gate/milestone meetings. The stage-gate secretary should keep a log of all meeting activities.

19.5.1 Committee Meetings

The stage-gate committee should meet at least quarterly, and invitees should include the committee, all project team members, and, as appropriate for the project, representatives from Legal, R&D, safety, and other functional areas. The meeting agenda includes:

1. Screen ideas.
2. Review project status, milestones, human resource needs, and budgets.
3. Set project priorities and allocate resource.

(1) Idea Vetting
The stage-gate committee should review ideas at least once per quarter, and additional meetings should be scheduled as needed. The frequency of idea reviews depends on the number of ideas submitted, the urgency of action needed on particular ideas, and staffing capacity to act on the ideas.

The committee may take the following actions on ideas:

1. Move the idea to Stage 2.
2. Return the idea to the submitter for clarification.
3. Hold the idea for future action.
4. Close the idea (no foreseeable future action).
5. Transfer the idea to another functional area.
6. Others (e.g., merge with an existing project).

If moving the idea to Stage 2 is selected, the stage-gate committee will assign a project team. If practical, the submitter of the idea should be included on the project team.

(2) Project Status Review
The purpose of the quarterly status review is to ensure that project timelines, milestones, human resource needs, budgets, and business strategic goals are being met. The meeting should be scheduled, the agenda created, and the meeting facilitated by the stage-gate secretary.

For each project in feasibility or beyond, short presentations (about 20 min) are given by the project leaders including the information outlined in the next section,

"Documentation, Project Summaries." Time should be allowed for discussion and scoring.

(3) Priority Setting and Resource Allocation
After all projects have been presented, the score sheets are totaled and a priority list created by point value within each stage and across stages. The committee should make adjustments to the ranking to create a balanced portfolio (e.g., risk and return, long- and short-term commercialization, product mix). Resources will be assigned to projects from top priority down until all resources are committed. Projects without resources will be put on hold until resources become available or priorities are changed. Personnel assigned to projects may change as a result or re-prioritization, although effort will be made to insure continuity whenever possible.

19.5.2 Gate/Milestone Meetings

Gate meetings are held when a project has completed a stage. Milestone meetings are held at decision-making points (for significant financial or human resource allocation or for major contractual commitments) within stages. In some cases, milestone decisions can be made by consensus through email.

Gate and milestone meetings are scheduled on an as-needed basis (i.e., when a project requires a gate or milestone decision) by the project leaders. Gate meetings are open to all executive leadership and closely affiliated personnel (e.g., IP and General Counsel). Other employees may attend with permission of the project leaders. The stage-gate committee will make a decision on the future status of the project based on the content of the project team's gate presentation, including the recommendation of the project team, and the discussion that follows.

Draft presentation decks should be sent to the committee and project team members at least 5 business days in advance of gate meetings. The Legal Department (IP and General Counsel) should be given copies of all decks. If significant changes have been made on the draft presentation deck, final presentation decks should be distributed at the gate or milestone meeting.

Questions, other than points of clarification, should be held until after the presentation. After the discussion ends, the committee can take one of the following actions:

1. Move to next stage or milestone.
2. Hold in stage for more information.
3. Close project.

In the event the committee is not able to reach consensus at a gate, the business unit leader's decision is final.

19.6 Documentation

19.6.1 Gate Presentations

The project team leaders should file the following after a gate or committee meeting:

1. Hard copy of final presentation deck and relevant supporting materials in the business central file system in project-specific, designated "stage-gate" folders
2. Electronic copy of final presentation deck on the business-shared drive

19.6.2 Meeting Logs

The stage-gate secretary should file the following after a gate/milestone or committee meeting:

1. Hard copy of the committee's decision and action list in the central file system in project-specific, designated "stage-gate" folders
2. Electronic copy of #1 on the business-shared drive
3. Updated project list, priority list, and/or idea log on the business-shared drive

19.6.3 Project Summaries

The following information should be updated quarterly in an electronic file on the shared drive ("project summaries") for each project in analysis or later stages. This update must be completed on the business day closest to the end of a given quarter:

- A succinct opportunity statement describing value creation
- Critical issues for success and brief plan for resolving them
- NPV (including list of assumptions on COGS, pricing, market size and expected penetration, volume); explanation for any changes in NPV from last quarterly review
- Human and financial resources required for the next milestones and remaining gates
- Timeline for the next milestones and remaining gates (Gantt charts)
- Technical risk score
- Commercial risk score
- Competitive advantages and threats
- Importance of speed to market to the project's success

19.7 Summary

Best practice for commercializing technology is to use a disciplined process for guiding the idea through the many stages that it will encounter throughout the process. This chapter summarizes both the stages and the different tools necessary for successfully navigating this effort. External resources, such as engineering firms, will usually offer their protocol which is similar to the one depicted here, albeit with different names and perhaps different number of stages. Regardless of which process one uses, adhering to the process of choice will increase the likelihood of the project being successful.