METABOLIC ENGINEERING IN METHANOTROPHIC BACTERIA

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ABSTRACT

Methane, as natural gas or biogas, is the least expensive source of carbon for (bio)chemical synthesis. Scalable biological upgrading of this simple alkane to chemicals and fuels can bring new sustainable solutions to a number of industries with large environmental footprints, such as natural gas/petroleum production, landfills, wastewater treatment, and livestock. Microbial biocatalysis with methane as a feedstock has been pursued off and on for almost a half century, with little enduring success. Today, biological engineering and systems biology provide new opportunities for metabolic system modulation and give new optimism to the concept of a methane-based bio-industry. Here we present an overview of the most recent advances pertaining to metabolic engineering of microbial methane utilization. Some ideas concerning metabolic improvements for production of acetyl-CoA and pyruvate, two main precursors for bioconversion, are presented. We also discuss main gaps in the current knowledge of aerobic methane utilization, which must be solved in order to release the full potential of methane-based biosystems.

Keywords: natural gas, methanotroph, metabolic engineering

1. Introduction

1.1. Overview of methanotrophs

In order to provide context for the metabolic engineering sections of this review, we include a brief overview of methanotrophs and methanotrophy. Methanotrophs are bacteria that grow on methane as their sole carbon and energy source. A resurgence in interest in these bacteria is occurring, in part due to interest in mitigating methane in the atmosphere as a greenhouse gas (Shindell et al., 2012) and in part due to the abundance and low cost of natural gas and its potential to create liquid value-added products (Conrado and Gonzalez, 2014). The latter processes have the potential to play a role in future energy sustainability. In this review, we will focus on those bacteria that depend on O₂ to oxidize methane.

Figure 1, below, provides an overview of aerobic methanotrophs and their metabolism. The reader is referred to a website that contains a great deal of basic information on methanotrophs (<http://www.methanotroph.org>), from which this overview has been adapted.

Microbial utilization of methane is known to occur in both aerobic and anaerobic environments. Aerobic methanotrophs can be separated into three major groups: Group I (Gammaproteobacteria; also referred to as Type I and Type X; Anthony, 1982; Semrau et al., 2010), Group II (Alphaproteobacteria, also referred to as Type II and Type III; Dedysh et al., 2001), and Group III (Verrucomicrobia, sometimes referred to as Type IV (Murrell and Jetten, 2009)). This proposal will mostly focus on Group I methanotrophs, due to a number of advantageous metabolic capabilities. These methanotrophs condense formaldehyde with ribulose monophosphate, resulting in production of fructose-6-phosphate (Anthony, 1982; Semrau et al., 2010; Kalyuzhnaya et al., 2013). Once generated, fructose-6-phosphate is incorporated into core “sugar”-linked metabolic pathways, such as oxidative glycolysis, oxidative and non-oxidative pentose-phosphate pathways and the Entner-Doudoroff pathway (Trotsenko and Murrell, 2008; Kalyuzhnaya et al., 2013). Because these high flux sugar-phosphate dependent metabolic pathways are similar to those in current industrial strains such as *E. coli* and *S. cerevisiae*, the Group I methanotrophs could be envisioned as microbial catalysts that can substitute methane for sugars as a carbon feedstock.

All known aerobic methanotrophs use methane monooxygenase (MMO) for the first oxidation step that converts methane into methanol (Semrau et al, 2010; Fig. 1). Methanol is

oxidized to formaldehyde, which can then be converted into biomass or further oxidized to formate and then into carbon dioxide. Two iso-enzymes of MMO are known: soluble MMO (sMMO), which is found in only a subset of known methanotrophs, and membrane bound (or particulate) MMO (pMMO), which is found in almost all known methanotrophs. Both the sMMO and pMMO are mixed function oxidases, in which one atom from O₂ goes to methanol and the other to water, requiring the input of 2 electrons and 2 protons (Semrau et al, 2010). The sMMO uses NADH, but the physiological electron donor to the pMMO is still not known. Purification of pMMO results in a substantial loss of activity, and thus kinetic parameters and the natural electron donor of the enzyme are not well established. However, cultures expressing pMMO typically show higher affinity toward methane when compared to cells expressing sMMO. Furthermore, it has been shown that cells using pMMO for growth display higher growth yield, suggesting that the pMMO is the more efficient system for methane oxidation (Leak and Dalton, 1986). pMMO is located in specialized internal membrane structures, called ICMs (intracytoplasmic membranes; Anthony, 1982; Semrau et al., 2010).

1.2 Unsolved problems in methanotrophy.

Although methanotrophs have been studied for decades, major gaps still exist in our fundamental knowledge of this important microbial group, which have the potential to undermine metabolic engineering strategies. For successful metabolic engineering, it is important to understand what is not known about methanotrophy and how those knowledge gaps should be addressed. Examples of knowledge gaps are: we do not know the identity of the pMMO electron donor, the components of the broader methane oxidation system including those involved in electron transfer, or how carbon flux is regulated. In general metabolic pathways downstream from primary methane assimilation are poorly resolved, and little is known about how methanotrophic bacteria adjust to shifting environmental settings or cultivation conditions. These include the use of NH₄⁺ vs NO₃ or S²⁻ vs SO₄²⁻ as a nitrogen or sulfur source, respectively, as well as oxygen limitation and supplementation of growth with methanol, hydrogen or multicarbon organic compounds, etc. These gaps in our knowledge make it difficult to create useful metabolic models or predict key targets for metabolic engineering. Such problems must be resolved if the potential of methanotrophs to contribute to the energy economy is to be realized. Specific information regarding a set of unsolved problems is presented below.

pMMO electron donor. Despite a great deal of effort in this area, the physiological source of the electron donor to the pMMO is still not resolved (Fig. 2). Since the various possibilities result in significant predicted metabolic differences, especially with regard to any engineered pathway that involves NAD(P)H or ATP, this uncertainty needs to be resolved. Without a firm understanding of the NAD(P)H/ATP balance of the cell, predictive metabolic models cannot be trusted and a set of possible scenarios must be considered. Since metabolic models are one of the basic tools of the metabolic engineer, this lack of certainty is an important factor for successful metabolic engineering. Here we summarize current knowledge, to highlight the most likely scenarios that should be included in any metabolic system analysis.

The similarities between the pMMO and the ammonia monooxygenase (AMO; Holmes et al., 1995) have prompted assumptions that the two systems must have similar electron donors. Duroquinol has been shown to drive the pMMO *in vitro* (Cook et al. 2002; Choi et al., 2003; Shiemke et al., 2004) and in keeping with the AMO, it is assumed that the endogenous quinol (UQH₂) plays that role *in vivo* (Arp et al., 2007; Simon and Klotz, 2013). However, the source of electrons to reduce ubiquinone is still not clear. In analogy to the AMO system, it might be expected that electrons from methanol oxidation are used to reduced ubiquinone. The enzyme that oxidizes methanol in methanotrophs is the periplasmic PQQ-linked methanol dehydrogenase (MeDH), which is coupled to a cytochrome *c* (Anthony, 2004). Reverse electron transfer from the MeDH has been proposed, but is not fully supported by experimental data (Leak and Dalton, 1986). In some methanotrophs, a membrane-associated putative heme-containing formaldehyde dehydrogenase is present and has been suggested to be the source of electrons to generate ubiquinol (Semrau et al., 2010). However, genes encoding this formaldehyde dehydrogenase have been identified only in a few genomes of methanotrophs, suggesting that these enzymes are not the key drivers. One alternative that has been suggested is the type 2-NADH:quinone oxidoreductase (NDH-2) that is ubiquitous in methanotroph genomes (Choi et al., 2003).

Each of these proposed scenarios has differences in the predicted energy cost and resulting yields as well as the O₂/CH₄ consumption ratio, and should be considered separately in metabolic models. So far, existing experimental data on these parameters do not rule out any of these scenarios. The co-localization of pMMO and MeDH and reports of low O₂/CH₄ ratios appear to support the hypothesis of direct coupling from methanol oxidation (Fassel et al., 1992; Kitmitto et al., 2005; Culpepper and Rosenzweig, 2014). Alternative systems present for

formaldehyde and formate oxidation generate NAD(P)H, which could in turn be used to generate ubiquinol from ubiquinone (Trotsenko and Murrell, 2008; Vorholt 2002). In agreement with that theory, externally applied formate stimulates methane oxidation rates and can enhance methanol excretion (Furuto et al., 1999; Lontoh et al., 2000;). On the other hand, the ability of methanotrophic bacteria to convert methane into organic acids and H₂ upon oxygen limitation (described below), suggests that pMMO can use a source of electrons other than NADH (Kalyuzhnaya et al., 2013). In summary, although multiple hypotheses exist for the physiological electron donor for the pMMO, at this time none is clearly supported over the other. It is possible that depending on growth conditions, multiple sources of electrons could be brought into play, underscoring the importance of considering all of these possibilities when designing pathways that alter NAD(P)H or ATP pools in the cell.

Electron transport components. The analysis presented above highlights another gap in our knowledge, which is the makeup of the electron transport components in methanotrophs. Although assumptions can be made based on recognizable electron transport components in genome sequences, genetic, physiological and biochemical studies are needed to determine whether novel components exist that couple the oxidation of one-carbon compounds to energy metabolism. The lack of such experimental confirmation generates even more uncertainty in the NAD(P)H and ATP balance of the cell, and makes metabolic engineering approaches involving manipulation of the electron transport chain riskier than in bacteria in which such information is available.

Methane vs. methanol utilization. In methanotrophs, the cell yield is significantly higher on methane than on methanol (Whittenbury et al., 1970), even though the energy-using step of methane oxidation predicts the opposite result. This conundrum suggests that the methane oxidation system does not operate as currently predicted. A possible explanation could be that the pMMO interacts directly with the methanol dehydrogenase (MeDH), and this results in efficient energy coupling. It is known that in methanotrophs, the MeDH is located primarily in the lumen (periplasm) of the ICMs, loosely bound to the membrane surface, and in a complex with the pMMO (Culpepper and Rosenzweig, 2014), (Fig. 2). When the cells grow on methanol, pMMO and the ICMs are not generated, and the MeDH is located in the periplasm of the cytoplasmic membrane. It is possible that during growth on methane, the ICMs provide a structure that enhances complex formation between the pMMO and MeDH, which might enable

direct electron transfer from methanol oxidation to methane oxidation. This coupling may involve additional proteins and/or electron transfer components, but if so, they are not yet known. A recent study of interactions between pMMO and MeDH suggests the presence of an additional, unknown protein (Culpepper and Rosenzweig, 2014). Likely, resolution of this anomaly will help close the gaps noted above regarding fundamental bioenergetics in these bacteria.

Assimilatory pathways. The choice of which assimilatory pathway to consider for metabolic engineering depends to some extent on the end product desired, since high flux intermediates are different in the two pathways. The ribulose monophosphate cycle has mainly sugar-phosphate intermediates, while the serine cycle involves amino acids, CoA derivatives, and TCA cycle intermediates. Recent analysis of the ribulose monophosphate cycle in Group I methanotrophs (specifically *Methylobacterium* and *Methylobacillus* strains) showed that contrary to existing assumptions, a more efficient version of this cycle operates. ¹³C-labeling analysis showed that pyruvate is mainly synthesized via the Embden–Meyerhof–Parnas (EMP) pathway, as opposed to the Entner–Doudoroff (EDD) pathway, in cells grown in vials under methane and air (Kalyuzhnaya et al., 2013). However, it is possible there are conditions under which the relative ratio of these two pathways might change, and nothing is known about the dynamics of carbon flow under different nutritional conditions. This fundamental information is another gap in our knowledge that must be filled in order to be able to predict specific metabolic manipulations for biotechnological goals.

A number of recent studies on the obligate methanotroph *Methylosinus trichosporium* OB3b and the facultative methanotrophs *Methylocystis* sp. SB2 and *Methylocella silvestris* showed that serine cycle methanotrophs also are metabolically versatile (Yang et al., 2013; Matsen et al., 2013; Vorobev et al., 2014; Crombie and Murrell, 2014). It has been shown that operation of the serine cycle in those methanotrophs could be coupled with the ethylmalonyl-coenzyme A (EMC) pathway or the glyoxylate shunt (GS). ¹³C-labeling studies showed that a significant portion of the cell carbon (>60%) in such methanotrophs is derived from CO₂ (Yang et al., 2013). It could be predicted that in methanotrophs with the serine cycle/GS assimilation, CO₂ fixation contributes to at least 50% of cell carbon. Overall the metabolic arrangement is a relatively expensive route for C₁-assimilation and not surprisingly, all tested alphaproteobacterial methanotrophs show low growth yields on methane compared to the gammaproteobacterial methanotrophs (Table 1). This characteristic might suggest that attention for biotechnology

exploitation should focus on the gammaproteobacterial methanotrophs. However, the methanotrophic alphaproteobacteria offer a number of attractive metabolic properties for specific biotechnological applications. (1) This group has a relatively high flux through CoA-derivatives, such as acetyl-CoA, crotonyl-CoA, etc. which could be applied to biofuel production. (2) Since alphaproteobacteria co-utilize methane and CO₂, that group of microbes seems well-suited for biogas utilization. (3) Many alphaproteobacterial methanotrophs show very broad metabolic capabilities and can switch from C₁ to C₂-C₃ compounds (Dedysh and Dunfield, 2011; Semrau et al., 2011; Crombie and Murrell, 2014). This last characteristic provides specific advantages for the metabolic engineering of methane to methanol conversion, such as the potential to remove methanol dehydrogenase in these organisms. (4) Finally, the unique ability of *Methylocella silvestris* to metabolize C_n-alkanes could serve as a platform for construction of a microbial system for bioconversion of natural gas or liquefied petroleum gas with a high content of C₂-C₄ alkanes (Etiope and Ciccioli, 2009). Thus both alpha- and gammaproteobacterial methanotrophs have potential for industrial applications, depending on the desired result.

1.3 Methanotrophs vs. engineered strains for methane-based biocatalysis

To fully realize the potential of industrial biocatalysis of methane, it will be critical to choose the right production strain. Model industrial workhorses such as *E. coli* and yeast species have several benefits including rapid growth rate, large knowledge bases from long histories of strain development, and modern molecular methods for rapid metabolic engineering. However, expression of the full active pMMO methane oxidation machinery in a heterologous host has not been reported to date. Only the soluble domain of the beta subunit of pMMO has been expressed in *E. coli*, and in this case the activity is quite low (Balasubramanian et al., 2010). Furthermore, no repeatable methane oxidation activity has been detected for the sMMO when expressed in a non-methanotroph (Jahng et al., 1996; West et al., 1992). These results highlight the difficulties inherent in creating a non-methanotroph with the ability to metabolize methane and suggest a significant breakthrough will be required to achieve this goal. A recent report has detailed the use of an NAD-linked methanol oxidation system coupled to expression of the two key genes for the ribulose monophosphate pathway to achieve conversion of methanol to metabolic intermediates in *E. coli*, as assessed by ¹³C-labeling (Müller et al., 2015). This is an important step in achieving methanotrophy in a non-methylotroph, although so far, such efforts have not resulted in growth. It seems likely that some combination of high throughput combinatorial

genomic screening and selection will ultimately result in growth on methanol and later, growth on methane.

An alternative to a heterologous host is to utilize existing methanotrophic bacteria. Aerobic alpha- and gammaproteobacterial methanotrophs are genetically tractable, and some species have fairly rapid growth rates (e.g. 2-3 hour doubling time for *Methylomonas* sp.; Koffas et al., 2001). At least some gammaproteobacterial methanotrophs use the Embden Meyerhof Parnas pathway with a pyrophosphate-linked phosphofructokinase for glycolysis, which improves achievable carbon and energy yields compared to previous assessments that assumed Entner Douderoff pathway use (Kalyuzhnaya et al., 2013; Koffas et al., 2001). The requirement of input of reducing power in the methane oxidation reaction limits the theoretical energy yield of methane biocatalysis in aerobic methanotrophs to 64% (Haynes and Gonzalez, 2014). Furthermore, if reducing equivalents are growth limiting in these organisms, these electrons must be replaced by complete oxidation of some methane to CO₂, thereby further limiting theoretical carbon yield. Once again we can see that more information about the fundamental physiology of these organisms, including the source of the electrons for the pMMO, is required for accurate yield projections.

Several avenues are currently being explored to improve the theoretical energy and carbon yields of methane biocatalysis. These include protein engineering attempts that can be employed in native methanotrophs or heterologous hosts, such as modification of anaerobic enzymes including methyl-coenzyme M reductase (MCR) from archaea for use in reverse methanogenesis, or methylsuccinate synthase. For a detailed comparison of these strategies please see the recent review article by Haynes and Gonzalez (Haynes and Gonzalez, 2014). Thus, both the near term prospects of industrial biocatalysis of methane using aerobic methanotrophs as well as promising future technologies currently in development highlight the exciting new prospects for this field.

1.4 Examples of biotechnology involving methanotrophs.

The biotechnological potential of methanotrophic bacteria has been broadly discussed (Anthony, 1982; Trotsenko and Khmelenina, 2008; Smith and Murrell, 2009, 2010; Winder, 2010; Jiang et al., 2010; Fei et al., 2014). However only a few methane-based products have been made at the pilot- and commercial scales and so far, none of these have involved strains that

have been improved by metabolic engineering. Among these successful products are Single Cell Protein (SCP), which has been explored in the former USSR, UK, Norway and Denmark. Methanotrophic single cell protein has variously been trademarked as “Gaprin” (Egorov et al., 1990), “BioProtein” (Nofferm/Calysta) and “Uniprotein” (Unibio A/S). A few SCP plants operate on natural gas as a feedstock today, all of them focused on the nutritional market and livestock feed/additives (<http://www.unibio.dk>, <http://www.bioprotein.no>, <http://www.calysta.com>). The following constraints of methane-based production at the large/pilot scale have been outlined: (a) very high cost of the oxygen supply, which can contribute up to half of the whole production cost (Zimin, 2008); (b) high risk fermentation, as two high risk flammable gaseous compounds (O_2 and CH_4) are involved; (c) high level (up to 10% of biomass) of nucleic acids, which require additional treatment and removal for SCP (Yazdian et al., 2005); (d) culture-producer stability at low oxygen supply (Khmelenina et al., 1992); (e) a source of toxicity, due to accumulation of natural gas contaminants (n-alkanes and heavy metals) in cell biomass; (f) high risk of contamination during cell growth. This final point is one of the major challenges in the microbial SCP industry (Adedayo et al., 2011). Contamination of methane-based SCP processes has been found to be beneficial for the process rate (Bothe et al., 2002). On the other hand, contamination might negatively affect the culture, induce cell lysis, result in a bioreactor that will malfunction and crash, and could result in a product with characteristics less beneficial than the product approved through regulatory agencies..

Other products explored for production from methane are: poly(3-hydroxybutyrate (PHB), exopolysaccharides (EPS), methanol (Xin et al., 2004a,b; Park and Lee, 2013); amino acids, such as glutamate, alanine, and ectoine (Trotsenko and Khmelenina, 2008), biodiesel/lipids (Choi et al, 2011; Fei et al., 2014), fermentation broth and carotenoids (Koffas et al., 2005; Ye and Kelly, 2012). Group II methanotrophic cultures have displayed potential for production of monomer components, such as epoxides (Hou, 1984) and polymers such as poly- β -hydroxybutyrate (PHB) (Zhang et al., 2009). It has been demonstrated that Group II methanotrophic bacteria can accumulate PHB up to 50% of dry cell biomass (Doronina et al., 2008; Criddle et al., 2013). PHB production from methane is currently being explored by a number of companies in the US, Russia and India. The use of methanotrophic bacteria for the production of methanol at ambient temperature and atmospheric pressure from low-quality methane sources has attracted the

attention of many investigators (Corder et al., 1988; Labinger, 1995; Xin et al., 2004a,b; Cantrell et al., 2008; Park and Lee, 2013). Only Group II methanotrophs, usually strains of *Methylosinus trichosporium*, have been tested, and it was shown that the addition of CO₂ or direct inhibitors of MeDH activity can help to pause the reaction at the stage of methanol formation (Mehta et al., 1991; Furuto et al., 1999; Xin et al., 2004a). The theoretical feasibility of methanol production from methane and CO₂ has been demonstrated (Xin et al., 2004b). However, in general the final yield of methanol is quite low. So far, no attempts to enhance methane oxidation via genetic manipulation of methanotrophic cultures have been reported.

Among other biotechnological applications of methanotrophic bacteria are: methane emission control (Streese and Stegmann, 2003; Yoon et al., 2009; Huang et al., 2011), bioremediation (Semrau, 2011) and bioleaching (DiSpirito et al., 2011). Due to the low specificity of MMOs, certain types of contaminants, such as TCE, meta-chlorotoluene, phenol, chlorofluoro-benzenes, and mono- and dichlorobiphenyls, can be cometabolized by methanotrophs and removed from soils, sediments and ground water (Semrau, 2011). Electrolytic methanogenic/methanotrophic coupling provides a very promising approach for wastewater treatment (Guiot et al., 2008). Methanotroph-based biofilters are used in reduction of methane emission from landfills (Park et al., 2008; Scheutz et al., 2009). Due to the high copper requirement of methanotrophs expressing the particulate methane monooxygenase (pMMO), many species produce a chalkophore termed methanobactin (mb) that binds and reduces copper with extremely high affinity (10⁻²¹ M) (El Ghazouani et al., 2011; Kim et al., 2004). Mb's high binding affinity has made it appealing for many biotechnological applications, including as a therapeutic agent for controlling copper homeostasis in Wilson's disease patients (Summer et al., 2011; Zischka et al., 2011). Additionally, mb has been found to bind many other metals and will bind and reduce both trivalent gold and divalent mercury ions with a mechanism similar to that of copper ions, leading to their immobilization (Choi et al., 2006). This opens the possibility of using mb or mb-expressing methanotrophs for bioleaching with mining and environmental remediation applications. Additionally, mb can be used for the production of uniform copper and gold nanoparticles (Choi et al., 2006; DiSpirito et al., 2011; Vorobev et al., 2013). It was recently discovered that the mb precursor is a ribosomally produced polypeptide (Semrau et al., 2013). The fact that mb is genetically encoded means it may be possible to use mutagenesis to engineer

mbs with different metal specificities, thereby further enhancing the industrial potential of this versatile secondary metabolite.

2. Cultures and Genomes

A key aspect of metabolic engineering of methanotrophs is the range of cultures and genetic tools available. Below is a summary of these resources.

2.1. Cultures.

Many methanotrophic cultures have been isolated and formally characterized over the past 4 decades, starting with the classical study of Whittenbury, Phillips, and Wilkinson (Whittenbury et al., 1970). Currently, there are 22 formally described genera known (see www.methanotroph.org, Table 2.1 for an annotated list). As taxonomic techniques became more sophisticated, some of the strains that had been studied were renamed, making tracing literature for a specific strain difficult. Although the majority of known methanotrophs are obligate methylotrophs, i.e., able to grow only on one-carbon compounds (mainly methane and methanol), as noted above some methanotrophs are facultative, i.e., can utilize multi-carbon compounds for growth, mainly short-chain organic acids, ethanol and alkanes (Semrau et al., 2011; Dunfield and Dedysh, 2014). To date no methanotrophs have been shown to grow on sugars or rich media although it is common for methanotrophs to grow in mixed culture with heterotrophic bacteria. An excellent article by Dedysh and Dunfield (2011) outlines the procedures to ensure that any newly isolated methanotroph culture is not contaminated, either with a methanol-utilizer or a bacterium that utilizes multi-carbon compounds. The basis of the interactions found in such mixed cultures are not yet known.

Key biotechnology-relevant parameters, such as growth rate, yield and genetic tractability, have been reported for only a small subset of methanotrophic cultures. Thus it is not surprising that most of the metabolic engineering efforts today are focused on well characterized species such as *Methylococcus capsulatus* Bath, *Methylosinus trichosporium* OB3b and *Methylocystis* spp. However, recent efforts in culturing novel methanotrophic species have resulted in the isolation and characterization of a variety of strains that can provide a greater range of potential applications for biotechnology. A number of extremely thermophilic, psychrophilic, acidophilic, alkaliphilic, and halophilic methanotrophs have also been isolated, thus expanding the physiological range of aerobic methanotrophy (Kalyuzhnaya et al., 1999; Trotsenko and Murrell,

2008; Murrell, 2010). Growth parameters of methanotrophic cultures vary significantly and cover a broad range of pH (1-10), temperature (4 to 65°C), and salinity (0-10%) (Trotsenko and Khmelenina, 2008). As noted above, some newly described methanotrophic cultures (in the NC10 group) can oxidize methane even in the absence of oxygen (Ettwig et al., 2009). Since the primary enzyme for methane oxidation in these microbes is pMMO, the NC10 cultures could serve as a source of novel enzymes for methane bio-catalysis driven by intracellular conversion of N-species into O₂.

2.2 Genomes.

Genome sequences are now available for almost all genera of methanotrophic bacteria, either as complete or draft sequences (see www.methanotroph.org, Table 5.1). Most of these genomes have been analyzed under the auspices of OMeGA (Organization of Methanotroph Genome Analysis), a consortium focused on the sequencing and annotation of multiple methanotroph genomes. These genome sequences provide a rich resource for comparative analysis, and demonstrate that although core metabolic pathways appear similar, the genomes of relatively closely related strains have significant divergence. Sequenced genomes enable the deployment of systems biology approaches (gene expression analysis, proteomics, regulatory network reconstruction, metabolic network reconstruction and modeling) and are quickly becoming the foundation for more rigorous investigation of the metabolic potential and evolutionary aspects of methanotrophy (Kao et al., 2004; Murrell and Jetten, 2009; Khadem et al., 2011; Campbell et al., 2011; Yang et al., 2013; Kalyuzhnaya et al., 2013; Vorobev et al., 2014; Tamas et al., 2014).

3. Genetic Tools

Genetic tools are essential for metabolic engineering of an organism for industrial biocatalysis. To date, only aerobic methanotrophs of the alpha- and gammaproteobacterial classes have been found to be genetically tractable. Methods for genetic manipulation are similar to other model bacterial systems and will be described here briefly.

3.1. Introducing genetic material

Genetic material is primarily introduced into aerobic methanotrophs via conjugation with an *E. coli* donor. Vectors typically contain the RK2/RP4 origin of transfer for this purpose. Triparental

matings can be performed using both the donor strain containing the vector of interest as well as a strain harboring a helper plasmid such as pRK2013 to mobilize the vector (Figurski and Helinski, 1979). Biparental matings using the S17-1 donor strain, which contains chromosomally integrated helper genes for mobilization, are also common (Simon et al., 1983). Matings are performed on agar plates consisting of methanotroph medium such as nitrate mineral salts (NMS) supplemented with nutrients such as yeast extract to support the donor, and are incubated in an atmosphere containing air and methane. Conjugations are commonly performed for multiple days before selection, with efficiencies varying widely among methanotrophs. Many methanotrophs are resistant to 25 µg/mL naladixic acid, which can be used to purify the recipient strain from a nal-sensitive donor. Rifamycin-resistant methanotroph strains are also commonly selected and used for this purpose.

Electroporation has also been used to introduce genetic material into methanotrophs in some instances. Electroporation was used to introduce a suicide vector for deletion of the *pmo* operon into *Methylocystis* sp. st. SC2 (Baani and Liesack, 2008). A system for mutagenesis of *Methylocella silvestris* BL2 via electroporation of linear DNA fragments has also been reported (Crombie and Murrell, 2011). Development of electroporation protocols for additional methanotrophic species would significantly accelerate the use modern metabolic engineering techniques in these bacteria, and is an area under active investigation in multiple laboratories.

3.2 Insertion/deletion mutants

Insertions and deletions are commonly made in methanotrophs by marker exchange via standard homologous recombination methods using flanking regions of at least 500 base pairs of genomic sequence. Some frequently used vectors, such as pCM184, contain antibiotic markers flanked by *loxP* sequences to enable removal and reuse of the marker via the Cre recombinase (Marx and Lidstrom, 2002). Alternatively, unmarked mutants can be constructed using counterselection systems. Sucrose counterselection using a *sacB*-based system has been successfully used in multiple methanotrophic species including *Methylococcus capsulatus* Bath, *Methylomonas* sp. st. 16a, and *Methylobaculum buryatense* (Ye et al., 2007; Welander and Summons, 2012; Puri et al., 2015).

Transposon systems have also been used to introduce mutations into methanotroph genomes. A Tn5 system was also used to create nitrogen fixation mutants in *Methylosinus* sp. st. 6

(Toukdarian and Lidstrom, 1984). These methods are also useful for introducing diversity for selection and screening purposes. For example, a mini-Tn5 system containing promoterless carotenoid genes was used in *Methylobacter* sp. st. 16a to identify insertion mutants with increased carotenoid production.(Sharpe et al., 2007).

3.3 Replicating vectors

Broad host range replicating vectors have been used for gene expression in aerobic methanotrophic bacteria. Members of the IncP family of plasmids are commonly used, although other broad host range replicons such as pBBR and IncQ vectors have also been reported (Table 1). Replicating plasmids containing reporter genes are useful for rapid promoter probing. Reporters including GFP, dTomato, catechol dioxygenase (encoded by *xylE*), and beta-galactosidase (encoded by *lacZ*) have all been used successfully in methanotrophs, however cell-free extracts are often required for more accurate, repeatable results (Ali and Murrell, 2009; Ojala et al., 2011; Puri et al., 2015). A set of genetic tools therefore exists for performing all basic manipulations within aerobic methanotrophic bacteria, which can be applied to metabolic engineering efforts.

4. Metabolic modeling

Metabolic model simulations are widely used for identification of putative targets for genetic modification as well as for identifying potential influences of environmental or genetic perturbation on system behavior; or, ultimately, for *in silico* phenotype predictions that can guide bioengineering strategies (Lee et al., 2008; Zelle et al., 2008; Lee et al., 2012; Karr et al. 2012; Esvelt and Wang, 2013). A number of metabolic models focused on C1-metabolism, mostly methanol-utilization pathways, have been constructed (Van Dien and Lidstrom, 2002; Peyraud et al., 2011). The potential of metabolic modeling in environmental studies of methane cycling and methane biocatalysis has also been recognized for many years, leading to the development of a set of kinetic and methane utilization models (Sipkema et al., 2000; Yoon and Semrau, 2008). However, contrary to other C₁-based models, the theoretical calculation of methane utilization typically has shown very poor correlation with measured parameters (Leak and Dalton, 1986). Access to complete genome sequences of methanotrophic bacteria has provided new top-down approaches for initial metabolic reconstruction (Santos et al., 2011). A number of genome-scale biochemical network reconstructions of biotechnologically-relevant methanotrophic bacteria are

available in BioCyc (<http://www.biocyc.org>). However, those are based on automatic reconstructions, which should be carefully evaluated in accordance with published data for a microbe of interest (substrate consumption and biomass accumulation rates, biomass composition analysis, metabolic pathway validation via enzymatic activity, gene/protein expression) 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>) or PathwayTools (bioinformatics.ai.sri.com/ptools). In ideal situations, the reconstruction should be further validated through comparison of model predictions to phenotypic data. Two critical factors continue to hinder the development of comprehensive computational models: lack of fundamental knowledge on bioenergetics (e.g. source of energy to drive methane activation and components of the electron transport system) and the complex intracellular organization involving ICMs, which suggests compartmentalization of metabolic pathways. Once validated GSM-FBA models of well established microbial catalysts, such as *Methylococcus capsulatus* and *Methylosinus trichosporium*, are available, these will become powerful tools for engineering microbial strains to maximize output of selected metabolic byproducts in methanotrophic microbes.

5. Potential targets for metabolic engineering

A variety of products could potentially be made from methane using methanotrophic bacteria. Since methane is converted in high flux to pyruvate in Group I methanotrophs and acetyl-CoA in Group II methanotrophs, existing metabolic engineering strategies for generating products from those two intermediates could theoretically be “dropped-in” to methanotrophic metabolism (Fig. 3). In addition, in Group I methanotrophs, a variety of sugars are naturally synthesized at high levels in some strains (Fig. 3) including sucrose and glycogen (a glucose polymer), which opens the possibility to use methanotrophs to convert methane to a product that could then be utilized by an existing industrial strain (Eshinimaev et al., 2002; Koffas et al., 2005). Below, some examples of specific metabolic engineering approaches are discussed.

5.1. Improving flux into pyruvate and acetyl-CoA.

In order to develop an industrial strain that generates pyruvate- or acetyl-CoA-derived products, it will be desirable to improve flux into these key intermediates. A number of

modifications can be predicted as potentially providing benefit (Fig. 4). As glycogen and EPS are the main carbon sink/storage in Group I strains, the elimination of a few steps in their biosynthesis pathways should increase the pool of sugar-phosphates, which could be redirected to fuel/chemical biosynthesis needs. Furthermore, elimination of carbohydrate production might improve fermentation parameters, especially at low O_2 (Khmelenina et al 1992). A similar strategy could be applied to increasing the pool of acetyl-CoA in Group II methanotrophs, via elimination of PHB accumulation functions.

It has been shown that at low O_2 , methanotrophic cultures switch to a fermentation mode, resulting in a significant drop in cell yield due to excretion of formate, acetate, lactate and H_2 (Kalyuzhnaya et al., 2013). For products not involving these excreted compounds, elimination of acetate kinase and lactate dehydrogenase are obvious targets. Reduction of H_2 production is another key modification to increase the cellular pool of NADH. Most methanotrophic cultures possess at least one hydrogenase gene cluster, while some of them in addition have nitrogenase, which also could contribute to H_2 -production with a concomitant decrease in yield. Thus, unless N_2 is envisioned as a nitrogen source, the reduction of H_2 -excretion could be achieved via elimination of both systems.

The metabolic flexibility of Group I methanotrophs provides a number of solutions for improving the overall yield of targeted products. For example, the glycolytic pathway seems to be the main path for pyruvate production in all tested Group I methanotrophic species (Kalyuzhnaya et al., 2013). This route is predicted to generate 1 NADH and 1.7 ATP per 3 mol of formaldehyde converted into pyruvate. If targeted product biosynthesis does not require ATP or utilize NADH, the glycolytic path is not an optimal solution as it might lead to overproduction of ATP, requiring introduction of a futile cycle. One of the possible solutions is to increase flux through the Entner-Doudoroff pathway, which converts C_1 -carbon into pyruvate and NAD(P)H, without additional ATP release.

The main route for acetyl-CoA production in Group I methanotrophs is pyruvate dehydrogenase, and thus this step is linked with loss of carbon as CO_2 . Similarly to other microbial catalysts, the incorporation of synthetic non-oxidative glycolysis (Bogorad et al., 2013) might improve the carbon conversion efficiency, when the pathway operates in combination with EMP-variants of the RuMP pathway (Fig. 4). An alternative strategy to increase conversion efficiency of acetyl-

CoA production from methane could be interconnection of the serine cycle and RuMP cycle. Taking into account that all sequenced RuMP methanotrophs possess the majority of the serine cycle genes, metabolic improvement involving operation of both cycles might be easy to integrate and validate. It should be noted that while the production of acetyl-CoA from non-oxidative glycolysis or the serine cycle improves the overall carbon conversion efficiency of the process, it negatively impacts the NADH pool, and might not be a reasonable solution for products that would require a significant input of reducing power for biosynthesis.

5.2. Balancing O₂-linked methane oxidation and “fermentation-based biosynthesis”.

While a type of fermentation metabolism involving methane has been demonstrated for Group I methanotrophic bacteria, it is important to remember that the overall methane consumption process in these microbes depends on O₂ via the MMO. A simple switch in O₂ supply would be predicted to not provide an optimal solution, as O₂-limitation would dramatically impact overall system performance, and methane consumption would also be reduced. However, metabolic engineering should be able to provide the type of breakthroughs needed to utilize the fermentation capability of methanotrophs. Unfortunately very little is known concerning how methanotrophic cells switch from the respiratory mode to the fermentation mode and how methane oxidation is coupled to respiration. This is another area in which more knowledge is needed in order to move the field forward.

5.3. Production of fatty acid-derived compounds from methanotrophs

As noted above, both alpha- and gammaproteobacterial methanotrophs contain intracytoplasmic membrane systems that house the pMMO (Anthony, 1982; Semrau et al., 2010). These bacteria have therefore evolved to devote relatively high metabolic flux towards lipid production, making them attractive hosts for the synthesis of fatty acid-derived fuels from methane. Given the higher biomass yields for the Group I methanotrophs, they are more attractive candidates for this application than Group II strains. Because these species are genetically tractable, it should be possible to adopt strategies for metabolic engineering of fuel production that have been developed in model systems such as *E. coli*. One example is overexpression of the truncated cytoplasmic thioesterase *tesA* for free fatty acid production (Steen et al., 2010). This strategy has been applied in the cyanobacterium *Synechocystis* as well, thereby confirming its broad applicability (Liu et al., 2011). In addition, heterologous expression of the wax ester synthase

atfA may make it possible to produce fatty acid esters in some methanotrophic strains, as well as triacylglycerides if a diacylglycerol pool is present or generated (Janssen and Steinbuchel, 2014; Steen et al., 2010). Intriguingly, because methanol is generated from methane in methanotrophs, it may be possible to produce fatty acid methyl esters (FAMES) *in situ* without additional modifications for alcohol synthesis (Fassel et al., 1992). It is therefore promising that some *atfA* homologs have significant activity with methanol for FAME production (Lee and Hu, 2010). These examples demonstrate the exciting potential of aerobic methanotrophs as a platform for conversion of methane into versatile fatty acid-derived fuels.

5.4 Strategies for enhanced methane emission control.

Microbial methane mitigation strategies have been demonstrated by a number of studies (Nikiema et al., 2005; Yoon and Semrau, 2009; Huang et al., 2011). However clogging, high capital and operational cost of biofiltration systems remain challenging problems for field applications. It has been broadly discussed that a combination of biofiltration with production of value-added chemicals might stimulate deployment of the filtration units at emission sites. (Streese and Stegmann, 2003; Yoon and Semrau, 2009). The unique ability of halophilic methanotrophs to accumulate sucrose in response to dryness is an attractive approach for the development of a low-cost technology that does not require expensive pumps for water recirculation, eliminates clogging and produces a widely used sugar-feedstock (But et al, 2015; Kalyuzhnaya et al., 2015). Additional rational metabolic engineering of methanotrophic traits for improved sucrose excretion at semi-dry conditions might further increase the attractiveness of this mitigation technology.

Enhanced methane emission control will require construction of methanotrophic traits with properties beneficial for a specific mitigation site. For example wastewater treatment facilities (WWTF) around the world produce and typically flare methane as a part of the sludge decomposition process. The WWTF widely use alcohol (such as methanol or ethanol) to enhance biological nitrate and/or phosphate removal. Conversion of methane produced by the WWTF sludge digester into a carbon-feedstock might reduce the operational cost of WWTF. While construction of a methanol producing strain could be difficult, the construction of a methanotroph-variant producing ethanol is a feasible alternative. For example methanotrophic bacteria, such as *Methylobacterium alcaliphilum* 20Z can produce significant amounts of acetate

(Kalyuzhnaya et al., 2013). The elimination of acetate kinase and incorporation of aldehyde and alcohol dehydrogenase in such cultures has the potential to enable conversion of methane into ethanol.

6. Engineered communities for methane conversion

Engineered microbial consortia are a promising frontier in industrial biotechnology because they can be more robust than axenic cultures and enable the use of economically viable feedstocks (Jagmann and Philipp, 2014). An example application of synthetic microbial communities is the one-pot production of biofuels from lignocellulose, known as consolidated bioprocessing. Researchers have used naturally cellulolytic organisms in combination with an engineered production host such as yeast or *E. coli* to synthesize products including methyl halides and isobutanol from cellulosic biomass (Bayer et al., 2009; Minty et al., 2013). These types of strategies are advantageous because they enable modular product synthesis from non-glucose substrates using familiar production hosts that can be engineered using sophisticated genetic tools.

Aerobic methanotrophs naturally exist in microbial consortia and utilize and distribute carbon and energy from methane to non-methanotrophic heterotrophs. In enrichment cultures in which ¹³C methane is provided as the sole carbon and energy source, this label is rapidly distributed to non-methanotrophic species (Beck et al., 2013; Kalyuzhnaya et al., 2008). Furthermore, as noted above, in low O₂ environments the gammaproteobacterial methanotroph *Methylobacterium alcaliphilum* 20Z will secrete fermentation products including lactate, acetate, and H₂ (Kalyuzhnaya et al., 2013), which could feed engineered production strains such as *E. coli*. In industrial processes methanotrophic cultures may actually benefit from association with non-methanotrophic partners, as was seen during production of single cell protein over long periods of time using *Methylococcus capsulatus* Bath (Bothe et al., 2002). Some methanotrophic species also produce significant amounts (~30% dry cell weight) of glycogen which could subsequently serve as a substrate for hexose utilizing hosts for downstream product synthesis (Eshinimaev et al., 2002). These findings illustrate that aerobic methanotrophs are a promising biocatalyst for production of feedstocks from methane that can be used by other organisms.

Synthetic methanotrophic consortia may also enable more efficient strategies for methane oxidation that are only marginally thermodynamically favorable, such as anaerobic methane

oxidation via reverse methanogenesis (Haynes and Gonzalez, 2014). In marine environments, anaerobic methane oxidation is coupled to sulfate reduction in communities containing ANME archaea and sulfate-reducing Deltaproteobacteria, although the roles of each partner in these communities are still being investigated (Knittel and Boetius, 2009; Milucka et al., 2012). Overall, the use of methanotrophs in synthetic microbial consortia is a promising approach to harness methane as a cheap feedstock for industrial biotechnology. However, very little is known regarding the basis of methanotroph consortia and how those interactions could be manipulated, highlighting another opportunity for the future.

7. Summary and Future Directions

Methanotrophic bacteria and the machinery for converting methane into value-added products are both promising approaches for taking advantage of methane as a future biofeedstock. The availability of biogas makes methane a potentially renewable feedstock, and the current low price of natural gas provides impetus for short-term development based using this resource. As documented in this review, the tools are in hand to manipulate methanotrophs, and a set of new methanotrophic strains are now available to broaden the available metabolic characteristics for industrial biotechnology. In addition, recent advances in understanding methanotrophic physiology point the way to new opportunities for taking advantage of methanotrophic machinery. Despite these optimistic signs, a significant number of gaps in the fundamental knowledge of methanotrophy need to be filled to allow the potential of these systems to be fully reached. Since the new tools and systems approaches provide the opportunity to solve those problems, it seems likely that this field will see a number of significant breakthroughs within the next few years.

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951 Table 1. Selected examples of genetic tools used to study methanotrophy. For a more
 952 comprehensive list visit <http://www.methanotroph.org>
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Selected examples of replicating vectors used in methanotrophs				
Plasmid name	Replicon	Selectable marker	Species (Reference)	Notes
pVK100	RK2/RP4 (IncP)	Tc, Km	<i>Methylosinus trichosporium</i> OB3b (Lloyd <i>et al.</i> , 1999), <i>Methylobacterium album</i> BG8 (Nguyen and Chan, 2003)	
pSRK-Km	pBBR1	Km	<i>Methylococcus capsulatus</i> Bath (Welandar and Summons, 2012)	
pBHR1	pBBR1	Km, Cm	<i>Methylobacterium</i> sp. st. 16a (Sharpe <i>et al.</i> , 2007)	
pMHA200	RK2/RP4 (IncP)	Km	<i>Methylocella silvestris</i> BL2 (Theisen <i>et al.</i> , 2005), <i>Methylococcus capsulatus</i> Bath (Ali and Murrell, 2008)	Promoter probe vector containing <i>gfp</i> reporter gene.
pHM1	RSF1010 (IncQ)	Km, Strept	<i>Methylosinus trichosporium</i> OB3b (Lloyd <i>et al.</i> , 1999)	
pAWP78	RK2/RP4 (IncP)	Km	<i>Methylobacterium buryatense</i> (Puri <i>et al.</i> , 2015)	

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Selected examples of deletion/insertion vectors used in methanotrophs			
Plasmid name	Selectable marker	Species (Reference)	Notes
pAYC61	Km	<i>Methylobacterium album</i> BG8 (Berson and Lidstrom, 1997)	
pK18mob	Km	<i>Methylosinus trichosporium</i> OB3b (Stafford <i>et al.</i> , 2003), <i>Methylococcus capsulatus</i> Bath	Gm insertion cassette often added for gene disruption by marker exchange.

		(Csaki <i>et al.</i> , 2003)	
pCM184	Tc, Amp, Km insertion cassette	<i>Methylocella sylvestris</i> (Crombie and Murrell, 2014) <i>Methylomicrobium alcaliphilum</i> 20Z (Ojala <i>et al.</i> , 2011)	Inserts Km cassette flanked by <i>loxP</i> sites for marker recycling (not used in study)
pSUKSM	Km	<i>Methylomonas</i> sp. st. 16a (Ye <i>et al.</i> , 2007)	<i>sacB</i> sucrose counterselection system for unmarked allelic exchange
pJQ200SK	Gm	<i>Methylococcus capsulatus</i> Bath (Welanders and Summons, 2012)	<i>sacB</i> sucrose counterselection system for unmarked allelic exchange
pCM433kanT	Km	<i>Methylomicrobium buryatense</i> (Puri <i>et al.</i> , 2015)	<i>sacB</i> sucrose counterselection system for unmarked allelic exchange

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Figure Legends

Figure 1. Overview of methanotrophs and methanotrophic metabolism. Key cycles are circled in blue. Pathway abbreviations are boxed. H₄F: tetrahydrofolate pathway; H₄MPT: tetrahydromethanopterin pathway. Key enzymes are in blue: pMMO: particulate methane monooxygenase; sMMO: soluble methane monooxygenase; MeDH: methanol dehydrogenase; Hps: hexulose 6-phosphate synthase; Fdh: formate dehydrogenase; RuBisCO: Ribulose 1,5-bisphosphate carboxylase; SHMT: serine hydroxymethyltransferase.

Figure 2. Oxidation of methane by the particulate methane monooxygenase (pMMO). Locations in the cell are shown in red and key enzymes are in blue. The most likely physiological electron donor to the pMMO is ubiquinol (Q8H₂). The source of electrons to reduce ubiquinone to ubiquinol is not yet known. Methanol dehydrogenase (MeDH) is physically associated with the pMMO. The release of H⁺ in the periplasm contributes to the energetics.

Figure 3. Possible methane-based chemicals and fuels. Methanotrophic bacteria have the capacity to generate all key 1-, 2- and 3-carbon intermediates for these bioconversions. Virtually all biosynthetic modules for the production of advanced fuels or chemicals, developed for glucose-based fermentation in *E. coli*, could potentially be implemented in methane-utilizing strains.

Figure 4. Simplified map of the central metabolic pathways in Group I methanotrophic bacteria, such as *Methylobacterium* spp. and *Methylobacillus* spp. Key enzymes are shown in blue and pathway abbreviations are boxed. Green arrows: predicted metabolic alterations to improve carbon conversion efficiency for production of acetyl-CoA (AcCoA);

red Xs: suggested deletions that could lead to increased flux into pyruvate and AcCoA. SC:
serine cycle; H₄F: tetrahydrofolate pathway; H₄MPT: tetrahydromethanopterin pathway;
NOG: non-oxidative glycolysis pathway; EMP: Embden–Meyerhof–Parnas pathway;
EDD: Entner–Doudoroff pathway; PPP: pentose phosphate pathway. Xu5P: xylulose 5-
phosphate; F6P: fructose 6-phosphate; F1,6P: fructose 1,6-bisphosphate; G6P: glucose 6-
phosphate; GAP: glyceraldehyde 3-phosphate; Pi: inorganic phosphate; EPS:
exopolysaccharides. MMO: methane monooxygenase; MeDH: methanol dehydrogenase;
Fdh: formate dehydrogenase; Hps: hexulose 6-phosphate synthase; hpi: Hexulose 6-
phosphate isomerase; P_{Pi}-Pfk: pyrophosphate-dependent 6-phosphofructokinase;
GlgABC: glycogen production enzymes; ldh: Lactate dehydrogenase; ack: acetate kinase.

Figure 1

Figure 2

1018 **Figure 3.**

EMP Pathway
3Formaldehyde = Pyruvate + NADH + H⁺ + 1.7ATP
3Formaldehyde = AcCoA + CO₂ + 2NADH + 2H⁺ + 1.7ATP
ATP + 5P_i = 3PP_i + ADP

EDD Pathway
3Formaldehyde = Pyruvate + NADH
3Formaldehyde = AcCoA + CO₂ + 3NADH + 3H⁺

NOG Pathway
2Formaldehyde + 2PP_i = AcCoA

Serine Cycle
2Formaldehyde + 3ATP = AcCoA

Combination of EMP and NOG
10Formaldehyde = 4AcCoA + 2CO₂ + 4NADH + 4H⁺

Combination of EMP and Serine Cycle
4Formaldehyde = 2AcCoA + NADH + H⁺ + 1.3ATP