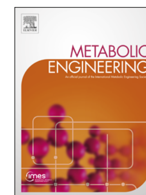
Metabolic Engineering 29 (2015) 142–152

Contents lists available at ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben



Metabolic engineering in methanotrophic bacteria

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ARTICLE INFO

Article history:
Received 24 October 2014
Received in revised form
26 February 2015
Accepted 17 March 2015
Available online 28 March 2015

Keywords:
Natural gas
Methanotroph
Metabolic engineering

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.

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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 O_2 to oxidize methane.

Fig. 1, 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 *Escherichia coli* and *Saccharomyces 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

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http://dx.doi.org/10.1016/j.ymben.2015.03.010
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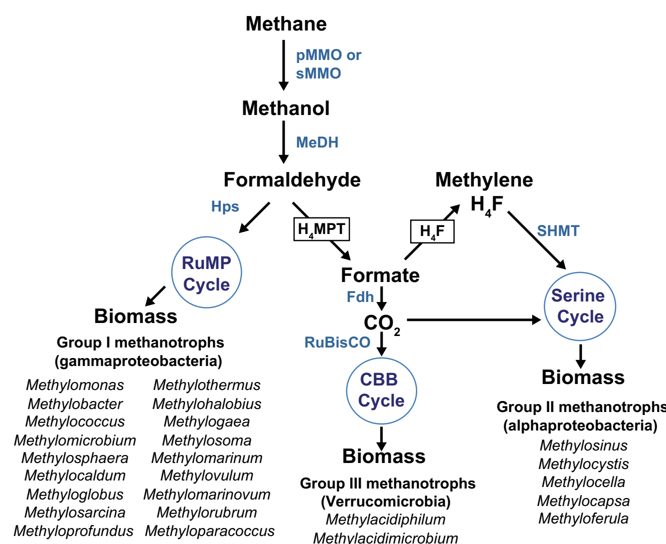

Fig. 1. Overview of methanotrophs and methanotrophic metabolism. Key cycles are circled in blue. Pathway abbreviations are boxed. H_4F: tetrahydrofolate pathway; H_4MPT: tetrahydromethanopterin pathway. Key enzymes are in blue: pMMO: particulate methane monooxygenase; sMMO: soluble methane monooxygenase; MeDH: methanol dehydrogenase; Hps: hexulose 6-phosphate synthase; Fdh: formate dehydrogenase; RuBisCO: Ribulose 1,5-bisphosphate carboxylase; SHMT: serine hydroxymethyltransferase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

function oxidases, in which one atom from O_2 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 NH_4^+ vs. NO_3^- or S^{2-} vs. SO_4^{2-} 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.

1.2.1. 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 and Shiemke, 2002; Choi et al., 2003; Shiemke et al., 2004) and in keeping with the AMO, it is assumed that the endogenous quinol (UQH_2) 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 ubiquonone. 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 O_2/CH_4 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 O_2/CH_4 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

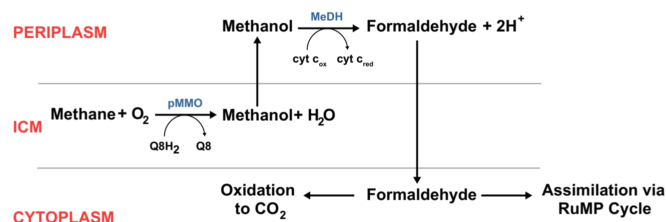

Fig. 2. Oxidation of methane by the particulate methane monooxygenase (pMMO). Locations in the cell are shown in red and key enzymes are in blue. The most likely physiological electron donor to the pMMO is ubiquinol ($Q8H_2$). The source of electrons to reduce ubiquinone to ubiquinol is not yet known. Methanol dehydrogenase (MeDH) is physically associated with the pMMO. The release of H+ in the periplasm contributes to the energetics. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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