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Contents

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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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.

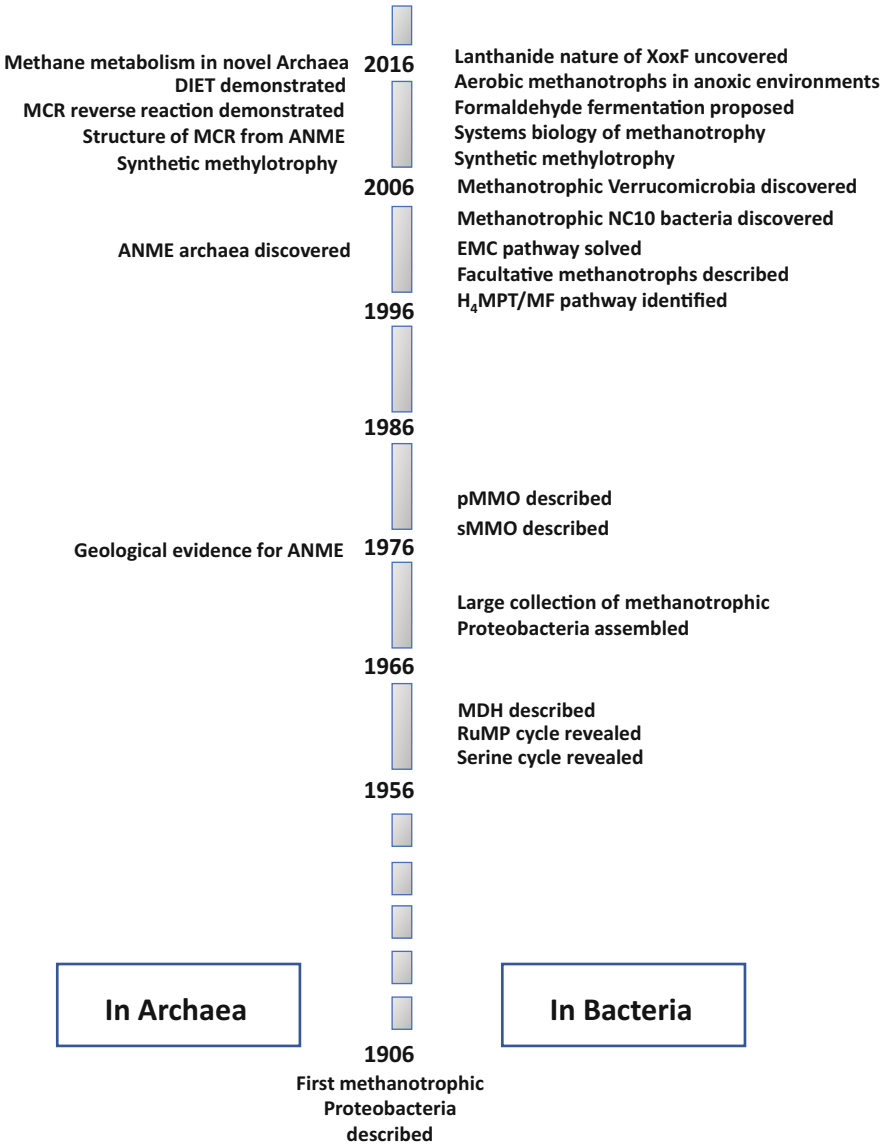

Fig. 1.1 Schematic of timeline for landmark discoveries in methanotrophy

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.

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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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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