# 2

# Diversity and Phylogeny of Described Aerobic Methanotrophs

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## 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*” | +/− |  |  |
| “*Methylacidimicrobium*” | “*M. fagopyrum*” | +/− | Acidophilic | van Teeseling et al. (2014) |
|  | “*M. cyclopophantes*” | +/− | Acidophilic |  |
|  | “*M. tartarophylax*” | +/− | Acidophilic |  |

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

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

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

Representatives of the genus *Methylomonas* are straight or slightly curved rods, occurring singly, in pairs, or in short chains. Most described species are motile by means of a single polar flagellum. These species often produce a surface pellicle in static liquid cultures. Production of red, pink, and orange carotenoid non-water-soluble pigments is highly typical for these methanotrophs. $C_1$ compounds are assimilated via the ribulose monophosphate pathway; ribulose-1,5-diphosphate carboxylase activity is absent. Some members of the genus can couple denitrification and methane oxidation. Several *Methylomonas* species fix dinitrogen via an oxygen-sensitive nitrogenase. Most representatives of the genus are mesophilic, growing between 10 and 40 °C. With the only exception of mildly acidophilic *M. paludis*, all described species are neutrophilic. Habitats are sediments of freshwater lakes and rivers, wetland muds, activated sludge and wastewater, coal mine drainage waters, and groundwater (Bowman 2016b).

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

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

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

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

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

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

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

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

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

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

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

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

### 2.3.2 Family *Methylothermaceae*

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

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

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

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

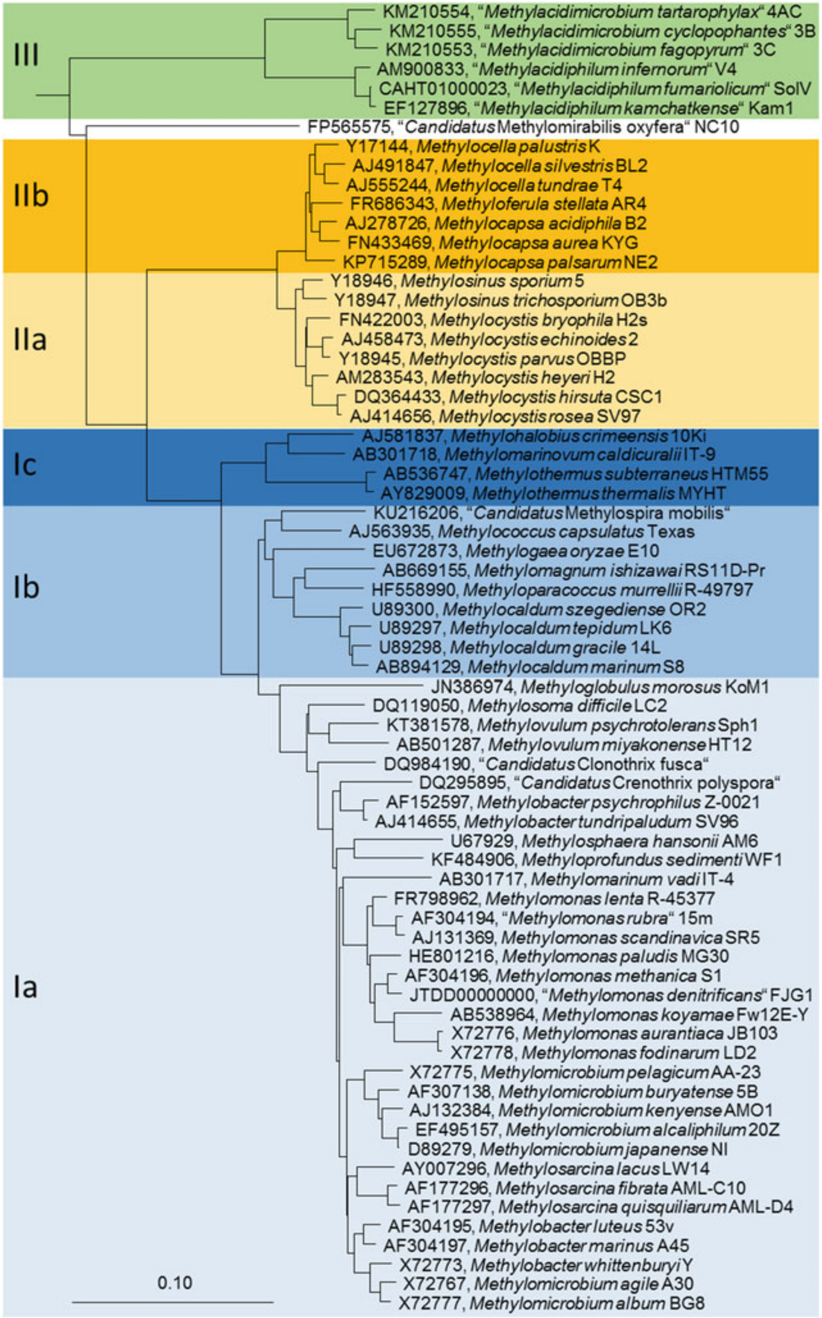

**Fig. 2.1** Phylogeny of described aerobic methanotrophic bacteria based on 16S rRNA gene sequences. A neighbor-joining tree was calculated with Jukes Cantor correction based on 1556 nucleotide positions using the ARB software package (Ludwig et al. 2004). The tree was rooted with sequences of methanogenic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

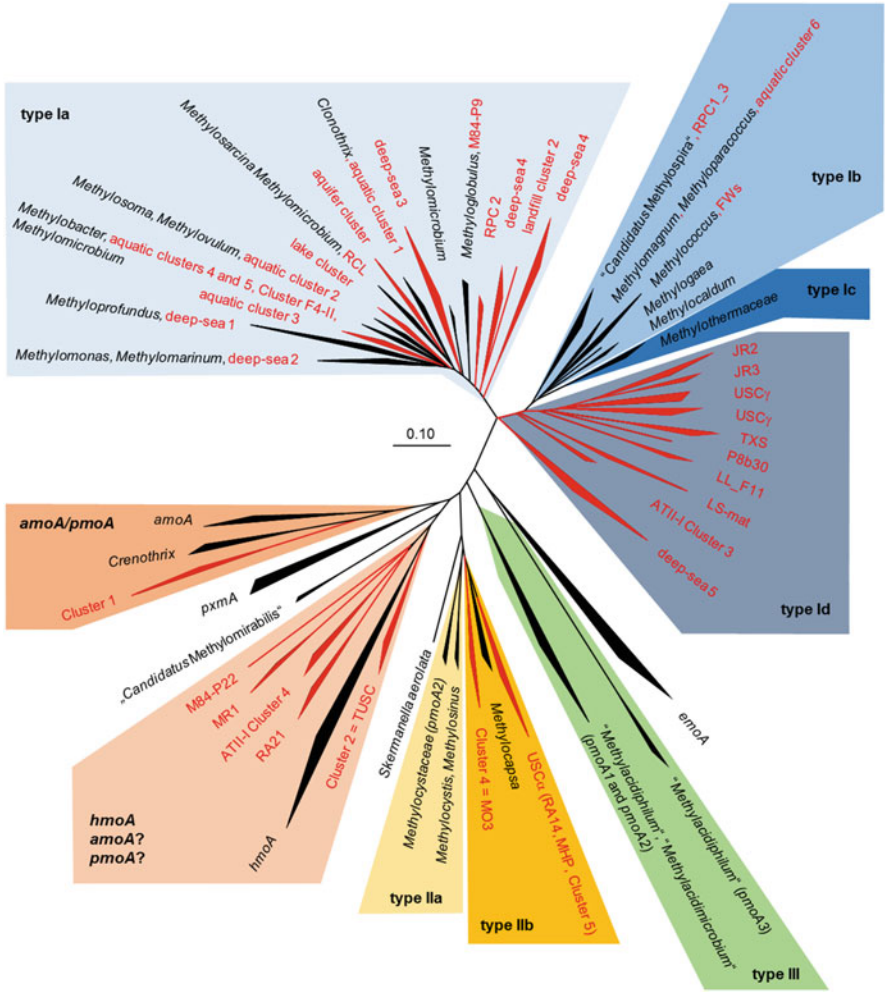

**Fig. 2.2** Radial neighbor-joining tree showing the *pmoA*-based phylogeny of uncultivated clusters of methanotrophs (highlighted in red) in relation to cultivated representatives (displayed in black). The tree was calculated based on 480 nucleotide positions with Jukes Cantor correction. The scale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

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

### 2.3.3 Family *Methylocystaceae*

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

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

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

### 2.3.4 Family *Beijerinckiaceae*

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

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

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

### 2.3.5 Methanotrophs of the Phylum *Verrucomicrobia*

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

### 2.3.6 Methanotrophic Bacteria with a *Candidatus* Status

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

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

## 2.4 Uncultivated Methanotrophic Bacteria

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

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

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

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

Several further clusters of *pmoA* sequences have been defined in the literature and are shown in Fig. 2.2. Their detection is mostly not limited to a specific habitat so that their ecological niche cannot yet be defined. They are mostly named after the name of a representative clone.

## 2.5 Final Remarks

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

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

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

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

(continued)

**Table 2.2** (continued)

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

(continued)

**Table 2.2** (continued)

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

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

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