Prokaryotes (2006) 5:266–289
DOI: 10.1007/0-387-30745-1_15

CHAPTER 3.1.14

The Methanotrophs—The Families Methylococcaceae and Methylocystaceae

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Introduction

Methanotrophs are a group of bacteria possessing a highly specialized metabolism restricted to the utilization of methane and methanol and are a subset of the methylotrophs, bacteria and archaea able to utilize C1 compounds. Methanotrophs are by definition obligately methylotrophic and do not have the ability to grow on organic compounds possessing carbon-carbon bonds. Besides methane, the only other substrate generally utilized by methanotrophs for growth is methanol; however, a few strains can utilize methylamine and a narrow selection of other C1 compounds. Methanotrophs are an integral part of the natural ecosystem, consuming much of the methane that is biogenically (through methanogenesis) and non-biogenically (e.g., from hydrocarbon seeps, natural gas fields and coal mines) derived. This interception of methane helps maintain a balance of atmospheric methane. Methanotrophs can utilize methane as they possess an enzyme called methane monooxygenase (MMO) which occurs, depending on the methanotroph species, either in a particulate (membrane-bound) or soluble (cytoplasmic) form. In a process referred to as dissimilatory methane oxidation, MMO oxidizes methane to methanol and then methanol is further oxidized to formaldehyde, which methanotrophs use for cellular carbon. Excess formaldehyde is oxidized to CO_2 via formate. The pathway also provides cells with reducing equivalents and drives electron transport for generation of ATP. The MMO is a powerful catalyzer of oxidation reactions and has been found able to oxidize a wide range of carbon compounds. This has given methanotrophs a significant biotechnological potential that has been harnessed in applications including bioremediation and industrial processes.

There are two major groups of methanotrophs, Type I and II. Type I methanotrophs are split into two more groups (Type I and Type X). The biology of Type I and Type II methanotrophs differs in phylogeny, chemotaxonomy, internal ultrastructure, carbon assimilation pathways, and certain other biochemical aspects. The differences are summarized in Table 1. The Type I methanotrophs are housed within the family Methylococcaceae which has six genera: *Methylococcus* (the type genus), *Methylocaldum*, *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosphaera*. The first two genera are also referred to as Type X methanotrophs, and this group is distinguished by certain physiological, biochemical and phylogenetic characteristics. The Type II methanotrophs are grouped in a family called the Methylocystaceae with *Methylocystis* (type genus) and *Methylosinus* as the member genera.

The taxonomy of the methanotrophs, which started with limited descriptions of several species groups, has undergone a series of developments (Whittenbury et al., 1970b). These studies were integral in advancing our understanding of methanotrophs. The subsequent usage of a systematic polyphasic approach (Murray et al., 1990) has resolved methanotrophic intra- and intergeneric relationships (Bowman et al., 1993b; Bowman et al., 1995). However it is clear the inherent biological and physiological diversity of methanotrophs remains untapped, as they appear to be ubiquitous in many environments. With the exploration of novel habitats using increasingly more sophisticated molecular techniques, the isolation of novel methanotrophs is inevitable. Thus, understanding of the role and ecology of methanotrophs and their intrinsic biology is still a highly active research area.

Phylogeny

The use of 16S ribosomal RNA-based phylogenetic analysis (Bratina et al., 1992; Brusseau et al., 1994; Bowman et al., 1995) has helped to resolve many nomenclatural problems which previously affected methanotrophs (Whittenbury and Krieg, 1984). On the basis of 16S rRNA sequence data, Type I methanotrophs belong in the gamma subdivision of the Proteobacteria; however, in most phylogenetic trees based on available sequences, the Family Methylococcaceae is made up of two separate groups (Figure

Table 1. Characteristics of methanotroph types.

Characteristics	Type I Methanotrophs	Type X Methanotrophs	Type II Methanotrophs
Family	*Methylococcaceae*		*Methylocystaceae*
Member genera	*Methylosphaera* *Methylobacter* *Methylomicrobium* *Methylomonas*	*Methylococcus* *Methylocaldum*	*Methylosinus* *Methylocystis*
Resting stages	*Azotobacter*-type cysts (or none)	*Azotobacter*-type cysts	Exospores or lipoidal cysts
Intracytoplasmic membranes	Type I	Type I	Type II
Soluble methane monooxygenase[b]	–[a]	–	+
Carbon assimilation pathway	RuMP	RuMP	Serine
Benson-Calvin cycle enzymes	–	+	–
Major fatty acid carbon chain length	16	16	18
Major quinone	Q-8 or MQ-8	MQ-8	Q-10
Mol% G+C (T_m)	43–60	56–65	60–67
Phylogenetic group (*Proteobacteria*)	Gamma	Gamma	Alpha

[a]Symbols: +, 90% or more of strains are positive; and –, 90% or more of strains are negative. RuMP pathway, ribulose monophosphate pathway.

[b]Absent in most Type I methanotrophs but is present in some strains of *Methylococcus* and *Methylomonas*.

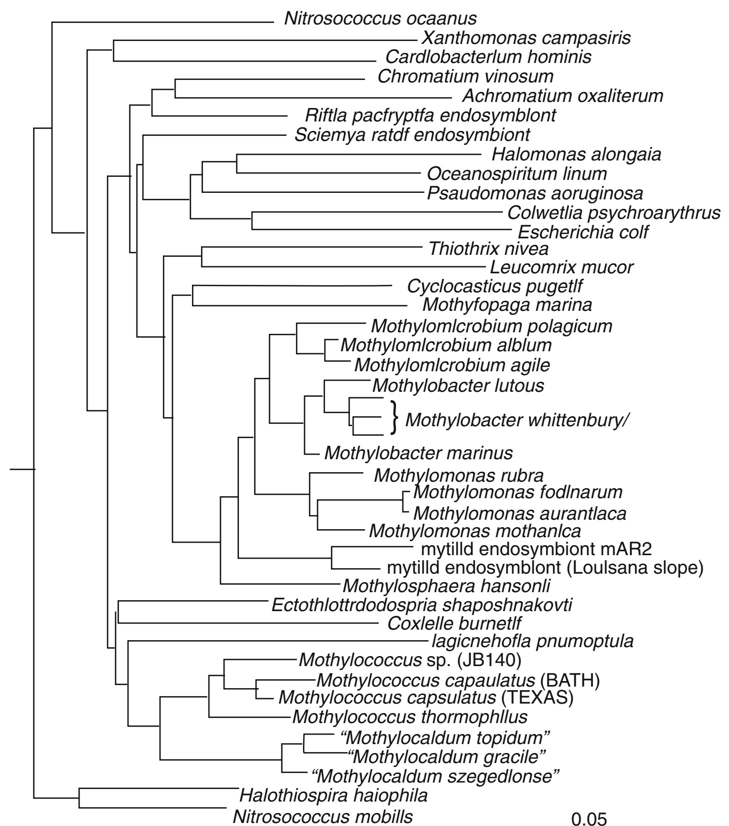

Fig. 1.

1). The first cluster includes the "Type X" methanotrophs—thermotolerant and thermophilic methanotrophs of the genera *Methylococcus* and *Methylocaldum*. This group deeply branches within the gamma subdivision, with the Ectothiorhodospiraceae, Chromatiaceae and Legionellaceae being amongst the closest relatives. The clade is separate from the remaining Type I methanotrophs, with evolutionary distances equal to other interposing genera. The second cluster contains mesophilic and psychrophilic type I methanotrophs, including the genera *Methylomonas*, *Methylobacter*, *Methylomicrobium* and *Methylosphaera*, as well as methanotrophic mytilid endosymbionts. This cluster is most closely related to *Methylophaga*

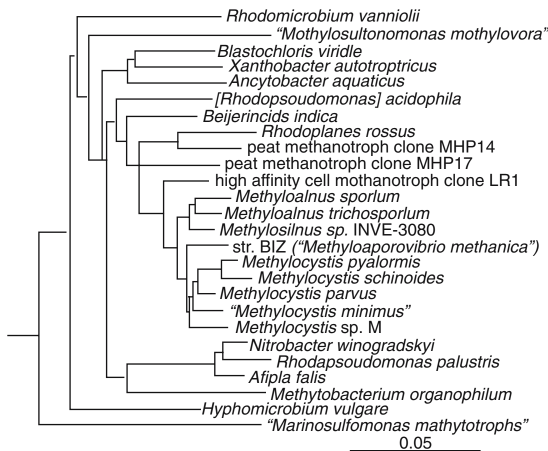

Fig. 2.

and *Cycloclasticus* and various chemoautotrophic endosymbionts.

Phylogenetic analysis based on 16S rRNA assigns *Methylocystis* and *Methylosinus* to the alpha subdivision of the Proteobacteria (Bratina et al., 1992; Brusseau et al., 1994). Owing to the close relationship, *Methylocystis* spp. is not clearly delineated from *Methylosinus* spp. (Figure 2). The Type II methanotrophs are more distantly related to an assemblage of facultatively methylotrophic, nitrogen-fixing and phototrophic bacteria including *Azorhizobium*, *Xanthobacter*, *Ancylobacter*, *Blastochloris*, *Rhodoplanes*, and *Beijerinckia*.

Taxonomy

Family Methylococcaceae

Genus Methylococcus and Genus Methylocaldum Both *Methylococcus* and *Methylocaldum* represent the Type X methanotrophs, a subset of Type I methanotrophs with a thermotolerant or thermophilic growth tendency. The type species of *Methylococcus* is *Methylococcus capsulatus*, which was originally described by Foster and Davis (1966) and included strains isolated from sewage sludge. Subsequent numerical taxonomic analyses suggested several of the methanotrophic species groups of Whittenbury et al. (1970b) were related to *Methylococcus*, and new *Methylococcus* species descriptions were later published (Romanovskaya et al., 1978) including the species *Methylococcus bovis*, *Methylococcus chroococcus*, *Methylococcus luteus*, *Methylococcus vinelandii* and *Methylococcus whittenburyi* ("*Methylobacter capsulatus*"). However, later investigations using immunological analysis, protein electrophoresis, fatty acid analysis and genomic characteristics (Andreev and Galchenko, 1978; Galchenko and Nesterov, 1981; Bezrukova et al., 1983; Meyer et al., 1986, Bowman et al., 1991a; Bowman et al., 1991b) clearly demonstrated the genus was made up of two groups. This nomenclatural problem was resolved when only *Methylococcus capsulatus* and *Methylococcus thermophilus* were retained in *Methylococcus* with the description of *Methylococcus* appropriately emended to reflect the change (Bowman et al., 1993b). The other species were transferred to the genus *Methylobacter*, the original name coined for them by Whittenbury et al. (1970b). Other species have been described and include "*Methylococcus fulvus*" (Malashenko et al., 1972), which appears to be a synonym of *Methylobacter luteus* (Romanovskaya et al., 1978), whereas the single strain of the species "*Methylococcus mobilis*" (Hazeu et al., 1980) unfortunately has been lost.

Moderately thermophilic methanotrophs were recently described by Bodrossy et al. (1997) as the genus *Methylocaldum*. This group includes the species *Methylocaldum szegediense* (the type species), *Methylocaldum tepidum* and *Methylocaldum gracile*. The latter species was renamed from "*Methylomonas gracilis*" NCIMB 11128. Bodrossy et al. (1997) noted phenotypic similarity between *Methylococcus thermophilus* and *Methylocaldum* spp., though they appear to be phylogenetically distinct (evolutionary distance ~0.08). However, high levels of DNA:DNA hybridization have been recorded between *Methylococcus thermophilus* IMV-2Yu and *Methylocaldum gracile* NCIMB 11912 (Bowman et al., 1993b). As 16S rRNA sequences for these strains are quite divergent, the 16S rRNA sequence of *Methylococcus thermophilus* needs to be verified.

Genus *Methylomonas* The type species of the genus *Methylomonas* is *Methylomonas methanica*. The genus was first described officially by Whittenbury and Krieg (1984); however, this species has been known for quite some time as it was first isolated by Soehngen (1906), who named it "*Bacillus methanica*," making it the first recorded methanotroph. Orla-Jenson (1909) subsequently renamed it "*Methanomonas methanica*." Morphololologically similar pink-pigmented strains were isolated also from methane:air enrichments of aquatic plants and other freshwater habits and were referred to as "*Pseudomonas methanica*" (Dworkin and Foster, 1956; Leadbetter and Foster, 1958). The name *Methylomonas methanica* was eventually coined by Whittenbury et al. (1970) for isolates very similiar to "*Pseudomonas methanica*," which they enriched from freshwater sediment.

Finally, the species was formally described by Romanovskaya et al. (1978), who at the same time described "*Methylomonas rubra*." Other pink-red pigmented groups of *Methylomonas* described by Whittenbury et al. (1970b) were regarded as variants of *Methylomonas methanica*. However DNA:DNA hybridization and phenotypic studies also suggested "*Methylomonas rubra*" was simply a synonym of *Methylomonas methanica* (Bowman et al., 1990). Additional species with a bright orange pigment isolated from coal-mine drainage water (*Methylomonas fodinarum*) and from sewage sludge and marshy soils (*Methylomonas aurantiaca*) also have been described (Bowman et al., 1990).

During the 1970s, with interest in biotechnology (particularly, the production of single-cell protein) expanding (Anthony, 1982; Hou, 1984), many methylotrophic bacteria were isolated which were grouped in the genus *Methylomonas*. Most of these species were able to utilize methanol and methylamine but not methane. In addition, practically all of these species were clearly misclassified, often lacking formal descriptions. Admittedly at that time, formal taxonomic arrangements were not available for the classification of methylotrophic bacteria. Thankfully most of these nomenclatural problems have now been resolved. Several invalid *Methylomonas* spp. are now recognized as belonging to *Methylobacillus glycogenes* (Urakami and Komagata, 1986b), including "*Methylomonas (Methanomonas) methylovora*" (Kuono et al., 1973), "*Methylomonas methanolica*," "*Methylomonas espexii*," "*Methylomonas methanocatalesslica*," and "*Methylomonas methanofructolica*" (Urakami and Komagata, 1986a). "*Methylomonas clara*" (Faust et al., 1977) has been shown to belong to *Methylophilus methylotrophus* (Jenkins et al., 1987).

A variety of methanotrophs were also grouped in *Methylomonas*, including "*Methylomonas methaninitrificans*" (Davis et al., 1964) and "*Methylomonas methanooxidans*" (Brown and Strawinski, 1958), both of which probably belong to the genus *Methylosinus* (Whittenbury et al., 1970a; Whittenbury et al., 1970b). The species "*Methylomonas margaritae*" (Takeda et al., 1974) and "*Methylomonas flagellata*" (Morinaga et al., 1976) possess traits very similar to those of *Methylomicrobium agile*; however, neither species has extant cultures. The marine species *Methylomonas pelagica* (Sieburth et al., 1987) was initially transferred to the genus *Methylobacter* (Bowman et al., 1993b) and then to the genus *Methylomicrobium* (Bowman et al., 1995).

GENUS *METHYLOBACTER* The representative type species of *Methylobacter* is *Methylobacter luteus*. The genus *Methylobacter* was formed following the emendation of the genus *Methylococcus* (Bowman et al., 1993b), and *Methylobacter* species are equivalent to the similarly named group first coined by Whittenbury et al. (1970b). Two species, *Methylococcus vinelandii* and *Methylococcus chroococcus*, have been shown by DNA:DNA hybridization to be synonyms of *Methylobacter whittenburyi*, while *Methylococcus bovis* is a synonym of *Methylobacter luteus*. Polyphasic taxonomic analyses have further refined the genus; species that cannot form cysts and have different fatty acid profiles were moved into the genus *Methylomicrobium* (Bowman et al., 1995) [RG1]. Recently two species "*Methylobacter psychrophilus*" (Omelchenko et al., 1996) and "*Methylobacter alcaliphilus*" (Khmelenina et al., 1997) were proposed for classification as strains, having been isolated from tundra soil and soda lake habitats, respectively. The genus contains the following validly described species: *Methylobacter luteus*, *Methylobacter whittenburyi*, and *Methylobacter marinus*.

GENUS *METHYLOMICROBIUM* The type species of *Methylomicrobium* is *Methylomicrobium agile* and it includes motile methanotrophs unable to form cysts. This genus was formed after polyphasic taxonomic analyses suggested that a number of *Methylobacter* species possessed sufficiently different traits (i.e., lack of encystment, more rod-like morphology, different fatty acid profile) to warrant the creation of a new genus (Bowman et al., 1995). The genus currently contains two terrestrial species, *Methylomicrobium agile* and *Methylomicrobium album*, and one marine species, *Methylomicrobium pelagicum,* previously called *Methylomonas pelagica* (Sieburth et al., 1987).

GENUS *METHYLOSPHAERA* A psychrophilic nonmotile, non-cyst-forming species from Antarctic lakes was found to form a distinct genus in the Methylococcaceae and is called *Methylosphaera hansonii* (Bowman et al., 1997).

Family Methylocystaceae

GENUS *METHYLOCYSTIS* The type species of the genus *Methylocystis* is *Methylocystis parvus*. The name *Methylocystis* was originally created by Whittenbury et al. (1970b); however, the actual description was published by Romanovskaya et al. (1978). Only *Methylocystis parvus* and "*Methylocystis minimus*" were included in the genus at the time, and several other species including "*Methylocystis methanolicus*," *Methylocystis echinoides* (Galchenko et al., 1977), "*Methylocystis pyreformis*," "*Methylocystis fuscus*" (Galchenko, 1977), and "*Methylocystis fistulosa*"

(Meyer, 1977) were ignored owing to lack of data, lack of a type strain and/or to the possibility that some were mixed cultures. The genus was not included in the Approved Lists of Bacterial Names (Skerman et al., 1980) but was subsequently revived and re-described in 1993 (Bowman et al., 1993b) and now includes *Methylocystis parvus* and the spinate, *Methylocystis echinoides*. Studies using protein electrophoresis (Galchenko and Nesterov, 1981) and immunotyping (Bezrukova et al., 1983) still suggest the diversity in *Methylocystis* is greater than what is currently recognized. Strains designated *Methylocystis echinoides* by Galchenko et al. (1977) were very similar to the strain IC 493S/5 (IMET 10491) of Haubold (1978) according to protein electrophoretic patterns (Galchenko and Nesterov, 1981). The strain IMET 10491 thus subsequently became the type strain of *Methylocystis echinoides* (Bowman et al., 1993b). "*Methylocystis minimus*" IMET 10519 (Whittenbury et al., 1970b; Romanovskaya et al., 1978) has been shown to be a synonym of *Methylocystis parvus* (Bowman et al., 1993b). Strain A of "*Methylovibrio soehngenii*" (Hazeu and Steenis, 1970) is probably just another strain of *Methylocystis parvus* (Anthony, 1982).

GENUS *METHYLOSINUS* The type species of the genus *Methylosinus* is *Methylosinus trichosporium*. As in the case of *Methylocystis*, the name *Methylosinus* was first coined by Whittenbury et al. (1970b) but was not published with a description until Romanovskaya et al. (1978) described the genus and two species, *Methylosinus trichosporium* and *Methylosinus sporium*. The genus was not included in the Approved Lists of Bacterial Names (Skerman et al., 1980) but was subsequently revived in 1993 (Bowman et al., 1993b). A variety of microorganisms have been confused with *Methylosinus* species including "*Blastobacter henrici*," which was misidentified as *Methylosinus trichosporium* (Trotsenko et al., 1989). "*Methylovibrio soehngenii*" strain B (Hazeu and Steenis, 1970) appeared to have a morphology identical to that of *Methylosinus sporium* (Anthony, 1982). Based on morphology, nitrogen-fixing methanotrophic strains designated "*Methylomonas methaninitrificans*" (Davis et al., 1964) and "*Methylomonas methanooxidans*" (Brown and Strawinski, 1958) are probably synonyms of *Methylosinus trichosporium* and *Methylosinus sporium*, respectively (Whittenbury and Krieg, 1984). Based on morphology and 16S rRNA sequences, "*Methylosporovibrio methanica*" strain 81Z (Tsuji et al., 1990) also appears to be equivalent to *Methylosinus sporium*. *Methylosinus*-like strains possessing a brilliant-red prodigiosin-like pigment (Strauss and Berger, 1983) also appear to belong to *Methylosinus sporium*.

Habitats

Methane is the next most important greenhouse gas after CO_2 and is the most abundant organic gas in the atmosphere. Methanotrophs' greatest significance is that they are the largest global methane sink and, as a result, are ubiquitous in nature. The largest and most active populations occur in environments with a stable gas exchange, in which both oxygen and methane are readily and continually available (Oremland and Cuthbertson, 1992; Reeburgh et al., 1993). Research indicates methanotrophs make up a high proportion of the total bacterial biomass in many freshwater aquatic environments, mostly within surface sediments (Boon et al., 1996; Ross et al., 1997). However their predominance in marine ecosystems is relatively unknown. Within these ecosystems methanotrophs represent a part of the basal trophic level and their carbon accumulates in the food web. Biogenic methane derived from methanogenesis is highly ^{13}C-depleted, and stable isotopic analyses indicate that carbon from methanotrophs reflects this. Measurement of ^{13}C isotope also suggests that a considerable proportion of carbon found in aquatic life at different trophic levels can be directly attributable to methanotrophic bacteria (Boschker et al., 1998). In other words, lower trophic levels such as zooplankton graze on methanotrophs and accumulate methanotroph carbon which is naturally ^{13}C-depleted, and as the zooplankton are consumed by higher life forms, this carbon can be traced up the food chain. Most generalist studies on methanotrophs in natural samples have measured methane oxidation rates as a very rough measure of population size and relative activity, however. Studies show in situ methane oxidation rates are affected by numerous biological and physical factors and are reviewed in some detail by Hanson and Hanson (1996).

Habitats in which methanotrophs are common include rice paddies; muddy soils of swamps and marshes; river, pond and lake surface sediments; meadow and deciduous forest soils; activated sewage sludge; some peat bogs; coal mine surfaces and drainage waters. Methanotrophs have in general not been isolated from "extreme" environments such as hypersaline lakes, hot springs and hydrothermal vents, even though some methanogenic archaea are able to grow in these habitats (e.g., *Methanopyrus*). For example, a study of a hypersaline mat environment failed to detect any methanotrophic activity (Conrad et al., 1995). However, methanotrophs have been isolated from Antarctic lakes, soda

lakes and tundra soils (Omelchenko et al., 1996; Khmelenina et al., 1997; Bowman et al., 1997) and thus some species possess psychrophilic and pH-based adaptations. In addition, methanotrophs (mostly of Type II) have been isolated from masonry of old buildings (Kussmaul et al., 1998).

Indirect immunofluoresence studies suggest Type I methanotrophs are common in surface sediments, the water column of various fresh and brackish water environments, and swampy soils (Reed and Dugan, 1978; Saralov et al., 1984; Ambramochkina et al., 1987; Malashenko et al., 1987; Galchenko et al., 1988; Galchenko, 1994). In addition 16S rRNA oligonucleotide signature probes (Brusseau et al., 1994) and isolation studies have demonstrated methanotrophs are associated with the roots and stems of aquatic macrophytes (Hanson et al., 1993; King, 1994; Calhoun and King, 1998). *Methylomonas* and *Methylococcus* spp. are found in significant populations in subtropical muds and swampy soils and in activated sludge (Bowman, 1992). *Methylococcus* and *Methylocaldum* strains have been isolated within and adjacent to thermal areas including thermal muds and springs which have temperatures up to 70°C or so (Malashenko et al., 1975a; Malashenko et al., 1975b; Bodrossy et al., 1997). The species "*Methylobacter psychrophilus*" was isolated from swampy tundra soils (Omelchenko et al., 1996), suggesting populations of methanotrophs in similar high latitude environments could be quite significant. In general little information exists on cold-adapted methane cyclers including both methanotrophs and methanogens. One place where such information does exist is Ace Lake in Eastern Antarctica, a marine-salinity meromictic water body in which methanogenesis occurs in the bottom water column and sediments (Franzmann et al., 1997). The lake supports a population of *Methylosphaera hansonii*, which concentrates in the oxycline of the lake (Bowman et al., 1997). It is possible this organism also may be common in surface sediments of the polar marine environments. Immunofluorescence studies suggest *Methylomicrobium pelagicum* is common in the upper mixing layers of temperate oceanic areas but is much less populous in estuarine waters (Sieburth et al., 1993). Conversely, *Methylobacter marinus* can be isolated from estuarine waters (Lidstrom, 1988). Various Type I methanotroph strains, intolerant to agar media (Lees et al., 1991), have been isolated from seawater off the coast of Britain.

Methylocystis and *Methylosinus* species have been isolated from a variety of soil and freshwater sediment habitats. *Methylosinus* strains tend to be isolated more regularly than *Methylocystis*, possibly because exospore formation makes them hardier. Indirect immunofluorescence studies suggest *Methylocystis* and *Methylosinus* can reach high populations in surface sediments of various freshwater and brackish water bodies (Reed and Dugan, 1978; Ambramochkina et al., 1987; Malashenko et al., 1987; Galchenko et al., 1988; Galchenko, 1994). *Methylosinus* has been found to dominate the culturable methanotroph population of groundwater (Bowman et al., 1993a) and rice paddy soils (Saralov and Babnazarov, 1982). Several strains of Type II methanotrophs have been isolated and detected on the root systems of the various aquatic macrophytes (Calhoun and King, 1998) and have been detected by gene probe methods in blanket peat bogs (McDonald et al., 1996).

Detection Direct plating procedures suggest methanotrophs are only a small proportion of the total viable microbial population in most samples. However direct plating methods are not very ideal for estimating methanotroph populations because of the poor transfer onto agar surfaces (usually less than 10% of cells survive subculture steps) and often rapid overgrowth by contaminating bacteria and fungi. Plating also is not aided by the intolerance to agar found in several methanotrophic strains from marine ecosystems. Most-probable-number counting based on liquid media, though generally labor- and materially intensive, gives a clearer indication of population levels (Bender and Conrad, 1994; Bowman et al., 1997; Escoffier et al., 1997) with the added advantage that methanotrophs can be isolated more easily from the highest dilutions.

Indirect immunofluorescence and ELISA techiques have been used extensively by research groups to detect specific species of methanotrophs, and the method appears useful in gaining a generalized concept of the distribution of methanotrophs (see above; Brigmon et al., 1998).

An alternative approach to detect methanotrophs involves use of specific phospholipid fatty acid (PLFA) biomarkers. For example, the PLFAs 16:1ω8c and 18:1ω8c (Nichols et al., 1985; Bowman et al., 1991a; Guckert et al., 1991) are very useful signatures for the detection and quantification of Type I and Type II methanotrophs, respectively, in environmental samples (Nichols et al., 1987; Sundh et al., 1995; Guezzenec and Fiali-Medioni, 1996; Boon et al., 1996). These fatty acids are practically methanotroph-specific. The detection of signature fatty acids relies on the confirmation of the double-bond geometry and isomeric state using gas chromatography-mass spectrometry. The quantity of the fatty acid also can be used to determine indirectly the biomass of methanotrophs in a given sample. However, a potential limitation

of this method is lack of sensitivity, as methanotroph populations in question must be relatively high (usually >1% of the total population) for lipid biomarkers to be reliably detectable. In addition, some methanotrophs, such as the species of *Methylococcus* and *Methylocaldum*, lack distinctive fatty acid biomarkers.

Nucleic acid oligonucleotide probe methods have been developed to detect methanotrophs in natural samples and have the advantage of detecting methanotrophs that have so far been resistant to cultivation, for example methanotrophic endosymbionts (Distel and Cavanaugh, 1994). Oligonucleotide probes have been developed from 16S rRNA sequences and shown to be specific for Type I and Type II methanotrophs (Brusseau et al., 1994). The method has been used for example to detect methanotrophs closely associated with aquatic plants such as duckweed (Hanson et al., 1993), freshwater lake water and sediment (Boon et al., 1996), peat (Dedysh et al., 1998; Edwards et al., 1998) and soil (Dunfield et al., 1999). A limitation of this method is that it depends on available sequence data and may underestimate populations that are present. To get around this problem gene probes based on methylotrophic functional genes have been developed also (Murrell et al., 1998). These include probes based on conserved genes of MMO and methanol dehydrogenase (McDonald and Murrell, 1997a; McDonald and Murrell, 1997b), which are known to be present in all methanotrophs and methylotrophs. Detection of these genes by PCR amplification has been useful in observing the distribution of methanotrophs in various environments including populations which so far have not been cultivated. For example methanotrophs detected in peat (McDonald et al., 1996) and seawater samples (Holmes et al., 1995b; Holmes et al., 1996) appear to belong to novel phylogenetic lineages distinct from other known methanotrophs. Finally more generalized molecular approaches for examining microbial communities can be applied to methanotroph studies. Jensen et al. (1998) utilized the DGGE (denaturing gradient gel electrophoresis) procedure to compare methane enrichments of agricultural soil and discovered that a number of novel groups of Type II methanotrophs were present. Also using DGGE, Dunfield et al. (1995) found a Type II methanotroph possessing a high affinity for methane and capable of oxidizing atmospheric methane (Bender and Conrad, 1992).

Endosymbionts Methanotrophic endosymbionts coexist with sulfur-oxidizing chemoautotrophic endosymbionts (Childress et al., 1986; Cavanaugh et al., 1987; Fisher et al., 1993) in a variety of unusual invertebrate communities associated with hydrocarbon seeps on the Pacific and Atlantic Ocean floor. Invertebrate hosts include deep-sea mytilid mussels (family Mytlidae; Childress et al., 1986) and the pogonophoran tubeworm *Siboglinum poseidoni* (Schmalijohann and Fluegel, 1987). Also cold water reefs of algae-free coral found in waters north of Norway, also associated with hydrocarbon seeps, are thought to use methane as a major nutrient and thus presumably contain methanotroph symbionts substituting for the algae (Hovland and Judd, 1988). That invertebrates can exist primarily on methane as a carbon source is shown by stable carbon isotope analyses (Southward et al., 1981; Cavanaugh, 1993). Methanotrophs growing in the gills of mytilids have been identified as Type I methanotrophs by the presence of key enzymes of the ribulose monophosphate pathway (RuMP), fatty acids, and hydrocarbons and by 16S rRNA oligonucleotide probes (Distel and Cavanaugh, 1994; Jahnke et al., 1995). Phylogenetic analyses indicate mytilid symbionts form a distinct lineage within the Methylococcaceae (Distel and Cavanaugh, 1994; Figure 1); however, cultivation of methanotroph symbionts has been unsuccessful to date. For more specific information consult the section of *The Prokaryotes* called "Symbiotic Associations between Prokaryotes and Animals" in Marine Chemesynthetic Symbioses in Volume 1.

Isolation and Cultivation

Media, Enrichment and Cultivation Methanotrophs can be enriched, isolated and cultivated in a mineral medium containing an inorganic nitrogen source and high purity methane in the headspace. The nitrate mineral salts (NMS) medium as described by Whittenbury et al. (1970a) is used with only minor modification of the mineral constituents. Nitrate salts are the usual nitrogen source for methanotroph cultivation; however, a low concentration of ammonia salts (<10 mM) can be substituted or used in combination with nitrates. High levels of ammonia are inhibitory to methanotrophs as it competitively inhibits MMO. Adding low levels of copper ensures good growth of all methanotrophs, as the membrane-bound form of MMO (see Physiology section) is a copper-containing enzyme. However, if copper is removed from the medium the isolation of methanotrophs able to form soluble MMO can be enhanced. Phosphates are also necessary for the growth of methanotrophs and in general media require 10–100 mM.

Unless otherwise specified, methanotrophs are always grown under methane. A small amount of sample is added to a liquid NMS medium (see below) in serum vials or in cotton-wool-stoppered flasks placed within air-tight contain-

ers. Agar plates can be incubated easily in containers, such as most desiccators and anaerobic jars, with an inlet tap or valve. An attached pressure gauge can be used if accurate methane additions are needed. Methane can then be added directly to vials and containers by first removing a portion of the headspace. The best methane : air ratio to use is equivocal but should be in a range of 1 : 10 to 1 : 1, as no dramatic difference in growth rates or yields occurs with the different ratios. Methane should be of high purity as natural gas could contain acetylene, which is a suicide substrate of MMO and will prevent growth even at very low concentrations. Static incubation proceeds at an appropriate incubation temperature. Most known species of methanotrophs, including those of *Methylocystis*, *Methylosinus*, *Methylomonas*, *Methylobacter* and *Methylomicrobium*, should be enriched and cultivated at 25–30°C. "*Methylobacter psychrophilus*" and *Methylosphaera hansonii* are psychrophiles, and their enrichment and cultivation should proceed at 2–10°C. In the case of the thermotolerant methanotrophs (*Methylococcus* and *Methylocaldum*), incubation should proceed at about 40–45°C, and depending on the source material, higher temperatures may be required. Enrichments take several days to several weeks, while pure cultures of most methanotrophs will form distinct colonies within 3–5 days. Some slow-growing species, such as *Methylosphaera hansonii*, may take up to 2 weeks to develop visible growth. Growth from the enrichments can then be directly plated onto mineral salts agar plates, which are then incubated under 1:1 methane:air or transferred to fresh liquid media for serial dilution. If samples contain large numbers of methanotrophs, turbidity in the enrichments will develop fairly rapidly (within 3–7 days) and static cultures often can develop a well-defined pellicle of growth, especially if *Methylomonas* species are enriched. For enrichments that rapidily develop turbidity, plating cultures early to achieve maximal biodiversity (if desired) is prudent, owing to the eventual domination of the culture by a single genotype. Unfortunately early plating does not make purification any easier. Pretreatment of samples can aid in the isolation of *Methylosinus* strains as they can be selected by exploiting the inherent heat- and desiccation resistance of their exospores. Soils and other samples should therefore be air-dried first and then heated to 80–85°C for 10–15 min before addition to enrichment media.

Methanotrophs also can be grown on methanol; however, this compound can often be toxic to methanotrophs due to accumulation of formaldehyde (see the Physiology section). Reliable growth on methanol can be achieved by incubating plates or liquid cultures in a sealed vessel containing a methanol-soaked tissue. The volatilized methanol is sufficient for growth. Alternatively, low levels of methanol can be used, though growth may be somewhat limited. Methanotrophs can be "trained" to tolerate higher levels of methanol by gradually building up the level of methanol (starting at 0.025% or so), with some strains able to tolerate levels as high as 5% (v/v). Specific strains of methanotrophs within a restricted number of species may use other C1 compounds (formate, methylamine, dimethylamine and formamide) for growth (see the Identification section).

Nitrate Mineral Salts Medium (NMS medium):

Component	Amount
$MgSO_4 \cdot 7H_2O$	1 g
KNO_3	1 g
$Na_2HPO_4 \cdot 12H_2O$	0.717 g
KH_2PO_4	0.272 g
$CaCl_2 \cdot 6H_2O$	0.2 g
Ferric ammonium EDTA	5 mg
Trace element solution	1 ml

Trace Element Solution:

Component	Amount
Disodium EDTA	0.5 g
$FeSO_4 \cdot 7H_2O$	0.2 g
H_3BO_3	0.03 g
$CoCl_2 \cdot 6H_2O$	0.02 g
$CuSO_4 \cdot 5H_2O$	0.03 g
$ZnSO_4 \cdot 7H_2O$	0.01 g
$MnCl_2 \cdot 4H_2O$	3 mg
$Na_2MoO_4 \cdot 2H_2O$	3 mg
$NiCl_2 \cdot 6H_2O$	2 mg

Preparation of trace element solution: Add components to distilled water and bring volume to 1 liter. Mix thoroughly. Neutralize pH with KOH.

Preparation of medium: Add components to 1 liter of distilled water (see below) and mix thoroughly. Adjust pH to 6.8 and distribute to culture vessels. If solid media are desired, add agar (Noble agar or agarose may also be used as well) to a concentration of 1.25 to 1.5% (w/v) and boil gently to dissolve the agar. Autoclave at 15 psi pressure (121°C) for 15 min to sterilize.

Artificial Sea Salts:

Component	Amount
NaCl	24.32 g
$MgCl_2$	5.143 g
Na_2SO_4	4.06 g
$CaCl_2$	1.14 g
KCl	0.69 g
$NaHCO_3$	0.2 g
KBr	0.1 g
H_3BO_4	0.027 g
$Sr(NO_3)_2$	0.026 g
NaF	0.003 g
Na_2SiO_3	0.002 g
$FePO_4$	0.002 g
NH_4NO_3	0.002 g

Preparation of artificial sea salts solution: Add all components to 1 liter of distilled water and dissolve thoroughly. A slight precipitation of calcium salts may be encountered but will not affect its use in media.

Media modifications: For growth of marine methanotrophs (such as *Methylomicrobium pelagicum* and *Methylosphaera hansoni*), the medium is prepared with sea salts (artificial or natural seawater) instead of distilled water, and pH is adjusted to about 7.5. A few methanotrophs prefer "brackish" media rather than seawater media including "*Methylobacter alkaliphilus*" for which the NMS medium is amended with 2% NaCl and the pH is adjusted to 9.0. The growth of *Methylobacter marinus* requires preparation of NMS medium with tap water and emendation with 1–2% NaCl and, after autoclaving, addition of 1 ml of vitamin solution. Oddly this species will not grow in media prepared with distilled water, even with added NaCl and vitamins.

Vitamin Solution:

Pyridoxine · HCl	10 mg
Calcium pantothenate	5 mg
Nicotinamide	5 mg
Nicotinic acid	5 mg
Riboflavin	5 mg
Thiamine · HCl	5 mg
Biotin	2 mg
Folic acid	2 mg

Preparation of vitamin solution: Dissolve components in 1 liter of distilled water and then filter (0.45-μm or 0.22-μm) sterilize the solution. For long-term storage, the solution should be refrigerated and shielded from light.

Other growth substrates: If methanol is desired it should be added only after the liquid medium has cooled to about room temperature. Agar media should be cooled to about 50°C before addition of methanol. The methanol should be filter-sterilized before use.

About 0.1% (w/v or v/v) formate, formamide methylamine, dimethylamine, trimethylamine, or trimethylamine N-oxide should be added as needed before autoclaving.

PURIFICATION One of the most problematic areas of methanotroph study is obtaining pure cultures. In practically all situations methanotroph enrichments are heavily contaminated by nonmethanotrophic (often methylotrophic) bacteria, which can easily overgrow and/or predate cultures. That is, predatory bacteria consume the methanotrophic bacteria, thus hampering isolation.

Because methanotrophs are relatively slow growers and plates are incubated in high humidity, fungal contamination is frequent unless containers are thoroughly cleaned with ethanol before each use. Combinations of fungicides such as cycloheximide and nystatin added to the medium are usually quite effective in reducing this problem. Cycloheximide and a suspension of nystatin can be added in a minimal amount of methanol (up to 0.25 ml per liter) to yield final concentrations of 200 U and 100 μg per ml of media, respectively. One approach to obtain pure cultures of methanotrophs is to use a plate microscope to observe colonies at an early stage of development (within 1–3 days). Colonies well separated from others can be picked with a sterile needle or loop and transferred to a clear section of the plate—well away from other developing microcolonies. Several colonies may have to be transferred in this way to obtain one successfully growing colony. It is necessary to make sure that the agar plates are fairly dry and excess liquid enrichment is not transferred onto the plate. This helps restrict the spreading of oligotrophic gliding bacteria and hyphomicrobia, both of which are a particular nuisance as they can rapidly overgrow the methanotroph colonies.

An alternative, rarely employed, straightforward approach to the isolation of methanotrophs utilizes NMS agar media containing a small amount of yeast extract (0.025% w/v) and methanol (0.025% v/v) (Malashenko et al., 1975) with incubation under a methane:air atmosphere. The enrichment cultures are serially diluted onto the media to the point of extinction. The yeast extract and methanol allow contaminants to reach larger colony sizes without affecting the growth of methanotrophs; indeed methanotroph growth may be considerably stimulated. Thus, methanotroph and contaminating bacterial colonies are more clearly visible. Single colonies on the spread plates are then transferred to liquid media. A number of passages from liquid media to spread plates and back to liquid media may be necessary. Several methanotrophs grow poorly or do not grow on agar. In some cases, highly purified agars, such as agar noble, used at lower concentrations may improve growth. Alternatively, silica gel may be used (Galchenko et al., 1975; Galchenko et al., 1977); however, preparation is often difficult and time consuming. For direct purification of these strains, a useful approach involves serially diluting in NMS liquid media in 96-well plastic titer trays (Bowman et al., 1997; Escoffier et al., 1997) as is done in most-probable-number counting experiments. Several strains can be purified in the same tray simultaneously. After sufficient incubation, the wells with the highest dilutions showing growth are examined microscopically. A number of separate transfers and dilutions may be required to obtain morphologically homogeneous cultures.

Some simple checks are necessary to assure the purity of methanotroph cultures. The checks include incubating methanotrophs on NMS agar or in liquid NMS media without methane; no growth should occur. In addition, strains should be plated in a complex organic medium such as nutrient agar (Oxoid or Difco) or R2A agar (Oxoid) and incubated with and without methane; again no growth should occur.

Identification

This section includes fairly detailed information on the morphology, ecophysiology, nutrition,

genotype and chemotaxonomy of methanotrophs (subdivided on the basis of family and genus) and can be used as a guide for identification. The information (unless otherwise indicated) comes from Bowman et al. (1993b). More specific information on metabolism and on physiologically related attributes, such as intracytoplasmic membranes and resting stages, can be found in the Physiology section. Characteristics differentiating the genera of the Methylococcaceae, e.g., Methylococcaceae and the Methylocystaceae, are summarized in Table 2 and Table 3, respectively.

FAMILY METHYLOCOCCACEAE *Genus Methylococcus* Cells of *Methylococcus capsulatus* are usually spherical and lack flagella. The cell envelope of *Methylococcus* species is typical for Gram-negative bacteria and cells are covered by an exopolysaccharide capsule (Whittenbury et al., 1970a) which often causes cells to clump. Single-bodied spherical cysts similar to those observed in *Azotobacter chroococcum*, but comparatively simpler and less defined in structure, are formed by *Methylococcus capsulatus* and *Methylococcus thermophilus* usually in the stationary growth phase (Whittenbury et al., 1970a; Malashenko et al., 1975b). *Methylococcus* strains also contain granules of poly-β-hydroxybutyrate, which contribute to changes in cell refractility. Cells also may contain polyphosphate (volutin) inclusions. Colonies on NMS agar are circular, convex, and smooth with an entire, even edge and have an off-white to pale tan pigment. *Methylococcus* spp. are thermotolerant or moderately thermophilic with optimal growth temperatures of 40–50°C, with no strains so far found that grow higher than 65°C or below about 25°C. No growth factors are required and the strains are non-halophilic. Some *Methylococcus capsulatus* strains can utilize methylamine, formate and/or formamide as sole carbon and energy sources. However, *Methylococcus thermophilus* strains, the type strain of *Methylococcus capsulatus* ATCC 19069 (strain "Texas"), and the more heavily studied strain ATCC 33009 (strain "Bath") cannot utilize any of these compounds. No carbon-carbon-bonded compounds can be used for growth. Nitrogen sources are usually provided in the form of a nitrate or ammonium salt, though *Methylococcus* strains can use yeast extract, casamino acids and amino acids. In addition, *Methylococcus capsulatus* and most strains of *Methylococcus thermophilus* can fix atmospheric nitrogen using an oxygen-sensitive nitrogenase (Murrell and Dalton, 1983b; Zhivotchenko et al., 1995).

The G+C content of the DNA of *Methylococcus capsulatus* ATCC 19069 has been found (62.5 mol%) using the buoyant density procedure. Analysis of several other strains using the thermal denaturation method found a range of 62 to 65 mol% (T_m). *Methylococcus thermophilus* strains have 59 to 61 mol% G+C (T_m), with the type strain IMV-2Yu possessing 59.1 (T_m; Table 3).

The major fatty acids in *Methylococcus* species have been found to be 16:0[16:0].

Fatty acid nomenclature: A fatty acid structure can be described as follows. The first digit indicates the number of carbon atoms in the fatty acid. Following the colon, the number indicates the number of double bonds present. Following this a symbol and digit indicate the position of the double bond from the methyl end of the fatty acid and is used to designate bonds from the carboxyl end. Suffixes including "c" and "t" indicate the isomeric form of the fatty acid, with "c" indicating a cis isomer and "t" a trans isomer. Prefixes may also be present to indicate structural modifications of the fatty acid. For example, "i" indicates iso-branching, "a" anteiso-branching, and "cy" that the terminal three methyl units have been cyclized. The prefix "x-OH" indicates the fatty acid is hydoxylated at the x carbon position. Thus, 16:1ω7c is a cis isomer with 16 carbon atoms and a single double bond positioned next to the 7th carbon from the methyl end (for fatty acid nomenclature see footnote). The high levels of 16:0 distinguish this species from psychrophilic and mesophilic Type I methanotrophs. The neutral lipid fraction of *Methylococcus capsulatus* is unusual for a prokaryote, containing squalene, methylated sterols and hopanoids (Bird et al., 1971; Neunlist and Rohmer, 1985). The unusual distribution of cyclic triterpenes and cyclopropane fatty acids (which increase with growth under reduced oxygen tension) are believed to improve the stability of outer- and intracytoplasmic membranes (Jahnke and Nichols, 1986; Jahnke et al., 1992). The major respiratory coenzyme Q in *Methylococcus capsulatus* and *Methylococcus thermophilus* is 18-methylene-ubiquinone-8 (MQ-8; Collins and Green, 1985). Detailed analysis of hydroxy fatty acids from the outer membrane lipopolysaccharide in various *Methylococcus* strains (Bowman et al., 1991a) revealed the major components are 3-OH 10:0, 3-OH 12:0, 3-OH 14:0, and 3-OH 16:0.

Genus Methylocaldum Strains of *Methylocaldum* (Bodrossy et al., 1997) appear as motile, rod-like to coccoidal cells, 0.5–1.2 µm wide and 1.0–1.8 µm long. In logarithmic phase, cells usually appear as short rods; however, in stationary phase, they are quite pleomorphic. Usually, spherical cells are present which possess *Azotobacter*-type cysts typical of other group I methanotrophs such as *Methylococcus*. Cysts

Table 2. Phenotypic characteristics differentiating member genera of the Family Methylococcaceae[a].

	Methylococcaceae[a]					
Characteristics	*Methylococcus*	*Methylocaldum*	*Methylosphaera*	*Methylobacter*	*Methylomicrobium*	*Methylomonas*
Cell morphology	Cocci-rods	Cocci-rods	Cocci	Cocci-ellipses	Rods	Rods
Motility	–[b]	+	–	D	+	+
Cyst formation	+	+	–	+	–	+
Desiccation resistance	–	D	–	+	–	–
Pigmentation:						
Carotenoids	–	–	–	–	–	+
Melanin-like	+/–	+	–	D	–	–
Other	–	–	–	D	–	–
Growth at 0°C	–	–	+	–	–	–
Growth at 25°C	–	–	–	+	+	+
Growth at 45°C	+	+	–	–	–	–
Requires seawater or Na† ions	–	–	+	D	D	–
RuBis CO[c]	+	+	–	–	–	–
Nitrogen fixation	+	–	+	–	–	D
Major fatty acids[d]	16:0, 16:lω7c	16:0, 16:lω7c	16:lω8c	16:lω7c	16:lω8c, 16:lω8c, 16:lω5t	16:lω8c, 14:0
Major quinone[e]	MQ-8	MQ-8	ND	Q-8	Q-8	MQ-8
Mol% G+C (T_m)	62–65	56–60	43–46	48–55	48–60	50–59

[a]Data from Bowman et al. (1993) and Bowman et al. (1997a).
[b]Symbols: +, 90% or more of strains are positive; –, 90% or more of strains are negative; D, result varies between species of the genus.
[c]Rubis CO, ribulose-1,5-bisphosphate carboxylase, key enzyme for the CO_1 fixation Benson-Calvin cycle.
[d]Fatty acids occur at greater than 10% of total phospholipid fatty acid content (Bowman et al., 1993, 1997).
[e]MO-8, 18-methylene-quinone-8; Q-8, quinone-8. Data from Collins and Green (1985).

Table 3. Differentiation of the Type II methanotrophs (Family *Methylocystaceae*) *Methylosinus* and *Methylocystis*.

Characteristics	*Methylosinus*	*Methylocystis*
Morphology	Pyriform or vibrioidal	Reniform to rod-like
Motility	+[a]	–
Exospores	+	–
Lipid cysts, spinae	–	D

[a]Symbols: +, 90% or more of strains are positive; –, 90% or more of strains are negative; D, result varies between species of the genus.

formed may or may not be heat resistant (able to tolerate 80°C for 20 min). In addition, thinner rods may be present in older cultures of *Methylocaldum gracile*. Cells are arranged singly, in pairs and, in the case of *Methylocaldum szegediense* and *Methylocaldum gracile*, in chains. All strains have typical Gram-negative cell walls and some strains may have extensive capsular material, in particular, strains of *Methylocaldum szegediense*. Colonies on NMS agar are circular, convex, smooth or rough, with an entire even edge, often have a cartilaginous consistency, and are pigmented tan to dark brown. At early stages of isolation a diffusible brown pigment is exuded into the agar media; however, this property is lost after repeated subculture.

Methylocaldum strains are obligately methanotrophic, growing best on methane as a sole carbon and energy source. Growth on methanol has not been obtained possibly because cells have a high level of sensitivity to formaldehyde. No growth occurs on other C1 compounds nor any compounds with carbon-carbon bonds. *Methylocaldum* strains can use either nitrate or ammonia as nitrogen sources; however, nitrogen fixation has been found to be absent. The genus includes strains that are mesophilic to thermophilic. *Methylocaldum gracile* and *Methylocaldum tepidum* can grow as low as 20°C and 30°C, respectively, and grow to a maximal temperature of 47°C, with an optimum of about 42°C. *Methylocaldum szegediense* can grow between 37 to 62°C, with an optimal growth temperature of 55°C. In other respects strains are neutrophilic and non-halophilic.

Methylocaldum strains possess mol% G+C values ranging from 56.5 to 57.2. Information on the chemotaxonomic properties is only available for *Methylocaldum gracile*, which overall appears to be similar to *Methylococcus*. The fatty acid profile is dominated by 16:0, 16:1ω7c and, depending on growth conditions, cy17:0. Also, the primary lipoquinone of this species has been found to be 18-methyleneubiquinone-8 (MQ-8; Collins and Green, 1985).

Genus Methylomonas The cells of *Methylomonas* species are regular-shaped rods, which are either straight or slightly curved, and they are also occasionally branched. Cells occur singly, in pairs, and sometimes as chains. All species are motile by a single unsheathed polar flagellum. They possess standard Gram-negative cell walls, well-defined slime capsules, and standard type I intracytoplasmic membranes. Cells contain simple single-bodied cysts similar to but better defined than cysts typically observed in *Azotobacter* and similar to those observed in *Methylococcus* species. In both species, cysts do not confer either desiccation or heat resistance. On NMS agar, colonies are circular, convex, and smooth, possess an entire edge and a creamy consistency and are pigmented either bright pink or orange. The colonies of *Methylomonas aurantiaca* have a more mucoid consistency and often segregate into rough-textured, cartilaginous variants.

Methylomonas spp. are obligate methanotrophs with sole carbon and energy sources restricted to methane and methanol; however, some *Methylomonas aurantiaca* strains also can utilize methylamine and more rarely dimethylamine. Other C_1 compounds that have been tested but are not utilized include formate, trimethylamine and trimethylamine N-oxide. Useable nitrogen sources include nitrate, ammonia, yeast extract, casamino acids, and various amino acids; however, the addition of complex nutrient sources such as yeast extract generally does not lead to growth stimulation and can be growth inhibitory at concentrations over 0.25% (w/v). Most *Methylomonas* strains can form a urease and use urea as a nitrogen source. *Methylomonas fodinarum* and *Methylomonas aurantiaca* are also able to fix atmospheric nitrogen by an oxygen-sensitive nitrogenase, while nitrogenase activity has only been detected in a few *Methylomonas methanica* strains. All *Methylomonas* species have a mesophilic and non-halophilic ecophysiology and grow between 10 and 42°C without the need for growth factors. *Methylomonas fodinarum* strains have temperature optima of about 25–30°C, while *Methylomonas methanica* and *Methylomonas aurantiaca* have temperature optima slightly higher, ranging from 30–35°C. The growth pH range is from 5.5 to 8.5 and best growth occurs at approximately pH 7.0.

The mol% G+C of the DNA of *Methylomonas* ranges from 50 to 59 (T_m). Fatty acid profiles of *Methylomonas* species have been found very similar, with 16:1ω8c, 14:0, 16:1ω7c and 16:1ω5t being the most abundant components. The relatively high levels of 14:0 and 16:1ω8c distinguish *Methylomonas* species from those of *Methylobacter* and *Methylomicrobium*. The fatty acids 16:1ω8c and 16:1ω5t are unusual and, beyond some other Type I methanotrophs, are practically unknown in bacteria and thus make excellent environmental signatures (see Ecology section).

The hydroxy fatty acids from the outer membrane lipopolysaccharide have been analyzed in detail in *Methylomonas methanica*, *Methylomonas fodinarum* and *Methylomonas aurantiaca* (Bowman et al., 1991a). The major component hydroxy fatty acid detected was 3-OH 16:0. Smaller proportions of 3-OH 12:0, 3-OH i13:0, and 2-OH 14:0 were also present, the levels of which vary considerably between the species. The primary respiratory lipoquinone has been identified as MQ-8 (Collins and Green, 1985).

Genus Methylobacter The cells of *Methylobacter* species possess a characteristic elliptical rod-like morphology with a width of 0.8–1.5 μm and a length of 1.2–3.0 μm and occur mostly singly or in pairs; however, chain formation is prevalent in some strains in the late exponential growth phase. *Methylobacter luteus* is nonmotile, while *Methylobacter whittenburyi* strains usually are motile when first isolated but can spontaneously lose the ability after extensive subculture. Motility appears most pronounced in young cultures of *Methylobacter whittenburyi* and *Methylobacter marinus,* with older cultures often devoid of motile cells. Motility is conferred by a single polar flagellum. Cells are surrounded by capsular material detectable by India ink staining, and cell walls are typical of Gram-negative bacteria. *Methylobacter* species form well-defined Azotobacter-type cysts which may give cells a refractile appearance (Whittenbury et al., 1970a). Poly-β-hydroxybutyrate granules tend to form in early log-phase cultures. Colonies on NMS agar are circular, convex, smooth, have an entire edge and possess a creamy consistency. The colonies of *Methylobacter luteus* are pigmented yellow and some strains form a diffusible yellow pigment. Both *Methylobacter whittenburyi* and *Methylobacter marinus* colonies are tanner and slowly exude tan to brown pigments into the agar media.

Methylobacter strains are strictly aerobic obligate methanotrophs with carbon and energy substrates limited to methane and methanol; however, a few strains can also utilize methylamine. Methane appears to be oxidized only by particulate MMO. The presence of soluble MMO (see Physiology section) has not been demonstrated in *Methylobacter* strains so far (Stainthorpe et al., 1991; Stainthorpe et al., 1991; Murrell et al., 1998). *Methylobacter* can utilize nitrate and ammonia salts, yeast extract, casamino acids and various amino acids as nitrogen sources. High levels (>0.5% w/v) of complex organic compounds are inhibitory to their growth. Some strains, particularly those of *Methylobacter luteus*, can produce a urease, but none are known to fix atmospheric nitrogen. *Methylobacter* species are mostly mesophilic, with most strains growing between 15 and 40°C and optimally at about 30°C. However, "*Methylobacter psychrophilus*" (Omelchenko et al., 1996) isolated from tundra is psychrophilic with a growth optimum of about 10°C and growth in the range of 0–20°C. In addition, this species is able to form gas vesicles. Methylobacter species are by nature neutrophilic with the pH range for growth ranging from 5.5 to 9.0 and a pH optimum at about 7.0. An exception is *"Methylobacter alcaliphilus"* (Khmelenina et al., 1997), which was isolated from a soda lake (in the Tuva region of Eastern Siberia) and grows best between 9.0–9.5 and also grows optimally with NaCl levels of 2–4%. Neither *Methylobacter luteus* nor *Methylobacter luteus* strains require growth factors, and they are non-halophilic. The estuarine species *Methylobacter marinus* grows optimally with about 0.1 M NaCl in tap water or with half-strength seawater salts. Some strains of *Methylobacter marinus* require nicotinic acid for growth (Lidstrom, 1988).

The mol% G+C of *Methylobacter* DNA ranges from 46 to 55 (T_m). *Methylobacter* species have very similar fatty acid profiles, with 16:1ω7c predominating and accompanied by lower levels of 14:0, 16:1ω6c, 16:1ω5c, and 16:0. The lack of 16:1ω8c and the relatively low levels of 14:0 and 16:0 distinguish *Methylobacter* from other Type I methanotrophs. The major fatty acids of "*Methylobacter alcaliphilus*" are 16:0, i16:0 and cy16:0, which differs from that of the other *Methylobacter* species (Khmelenina et al., 1997). The fatty-acid and polar-lipid profiles of this species vary considerably when grown under different cultivation conditions including pH and salinity. The outer membrane lipopolysaccharide hydroxy fatty acids have been analyzed in detail in *Methylobacter luteus* and *Methylobacter whittenburyi* (Bowman et al., 1991a). The major components found in *Methylobacter luteus* are 3-OH 10:0 and 3-OH 16:0 plus smaller quantities of 2-OH 12:0, 3-OH 14:0 and 3-OH 15:0, while the major component of *Methylobacter whittenburyi* is 3-OH 16:0, which is almost the only hydroxy fatty acid present. *Methylobacter* spp. contain Q-8 as their major respiratory lipoquinone (Collins and Green, 1985).

Genus Methylomicrobium Cells of *Methylomicrobium* spp. appear as single or paired, regular short rods, 0.5–0.1.5 μm wide and 1.5–2.5 μm long. All species are actively motile, propelled by a single polar flagellum, possess a standard Gram-negative cell wall, and are surrounded by a thin slime capsule. Cells contain type I intracytoplasmic membranes typical of other Methylococcaceae; however, they lack the ability to form cysts. Most strains contain poly-β-hydroxybutyrate and polyphosphate granules. Cells are not

heat- or desiccation resistant and are somewhat sensitive to methane starvation, losing viability in only a few days when exposed to a methane-free atmosphere. Colonies on NMS agar are nonpigmented, circular, convex-flat, and smooth and possess a creamy consistency.

Methylomicrobium strains are strictly aerobic obligate methanotrophs with carbon and energy substrates limited to methane and methanol; however, some strains of *Methylomicrobium agile* can use methylamine, dimethylamine and trimethylamine. Upon isolation most *Methylomicrobium* strains can tolerate and grow quite well on methanol. Methane appears to be oxidized predominantly by a particulate MMO (see Physiology section). The presence of soluble MMO has not been demonstrated in *Methylomicrobium* strains so far. *Methylomicrobium* spp. can utilize nitrate and ammonia salts, yeast extract, casamino acids, and various amino acids as nitrogen sources, though high levels (>0.5% w/v) of complex organic compounds are inhibitory to growth. Urease and nitrigen fixation activity is absent. *Methylomicrobium* species are mesophilic, growing between 10 and 30°C. *Methylomicrobium agile* and *Methylomicrobium album* grow best at 25–30°C, while *Methylomicrobium pelagicum* grows optimally at about 20–25°C. The pH range for growth is 6.0 to 9.0, with optimal growth occurring at about pH 7.0. Neither *Methylomicrobium agile* nor *Methylomicrobium album* requires growth factors, and they are non-halophilic. The marine species *Methylomicrobium pelagicum* grows optimally in media containing either natural or artificial seawater (Sieburth et al., 1987).

The mol% G+C of the DNA of *Methylomicrobium* is relatively broad, ranging from 48 to 60 (T_m). Fatty acid profiles of *Methylomicrobium* species are quite similar and the most abundant components are 16:1ω5c, 16:1ω5t, 16:1ω8c, 16:1ω7c and 16:0 (Table 2). The low levels of 14:0 and abundance of 16:1ω5t and 16:1ω8c distinguish *Methylomicrobium* from *Methylobacter* and *Methylomonas* species. The high levels of the trans fatty acid 16:1ω5t are unusual and do not appear to be due to stressful cultivation conditions. The carbohydrate fraction of the outer membrane lipopolysaccharide in *Methylomicrobium album* includes D-glucose, L-fucose, and D-heptose (Sutherland and Kennedy, 1986). The hydroxy fatty acids from the lipopolysaccharide fraction *Methylomicrobium album* and *Methylomicrobium agile* (Bowman et al., 1991a) are predominantly 3-OH 16:0. The primary respiratory lipoquinone is Q-8, as in Methylobacter spp. (Collins and Green, 1985).

Genus Methylosphaera *Methylosphaera hansonii* strains appear as featureless spherical cells, which exhibit refractility by phase contrast microscopy. Though cells may show signs of uneven binary division, evidence for budding division is still lacking. *Methylosphaera* cells possess standard Gram-negative cell walls and type I ICM when grown under methane; however, cells lack flagella, cysts or other types of resting stages typically found in methanotrophic bacteria. Cells actively accumulate poly-β-hydroxybutyrate, while preliminary electron microscopic examination indicates the presence of gas vesicles (dimensions 0.05–0.1 µm by 0.2 µm; Bowman et al, 1997; Bowman, unpublished data). The species is intolerant to all forms of agar and so far has only been grown successfully in liquid seawater-NMS media.

Methylosphaera hansonii uses methane and methanol as sources of carbon and energy, and no other C_1 or carbon-carbon bonded compound supports growth. Methane monooxygenase activity appears to be restricted to the particulate (pMMO) form with no soluble (sMMO) activity detected by the naphthalene oxidation assay. *Methylosphaera* strains can utilize nitrate, ammonia and L-glutamine for nitrogen; however, yeast extract and casamino acids are less suitable, causing partial inhibition of growth when tested at 0.05–0.1% (w/v) and complete inhibition of growth at concentrations of 0.25–0.5% (w/v). Though strains of *Methylosphaera* cannot form a urease, they can fix atmospheric nitrogen. All strains are psychrophilic with optimum and maximum growth temperatures varying slightly between strains. Optimal growth occurs at 10–15°C, while no growth occurs at 25°C. A doubling time of 20–24 hours was determined for strains growing at or close to their optimum temperature. In NMS-seawater liquid media, growth occurs at pH 6.0–8.0 and a pH of approximately 7.5 is optimal for growth.

The DNA base composition values of *Methylosphaera hansonii* are the lowest among the known methanotrophs, ranging from 43 to 46 mol%. The type strain AM6 (ACAM 549) possesses a mol% G+C of 44.8. The major fatty acids of the genus include 16:1ω8c, 16:1ω7c and 16:1ω6c. The lack of 16:1ω5c and 16:1ω5t is useful in differentiating the species from other Type I methanotrophs.

FAMILY METHYLOCYSTACEAE *Genus Methylocystis Methylocystis* strains typically appear as small, nonmotile kidney bean-shaped or rod-shaped cells (0.8–1.0 µm long, 0.4–0.5 µm wide) which divide by binary division. Several light microscopic photographs of *Methylocystis parvus* can be found in the article by Whittenbury et al. (1970a, 1970b). Cells are in a well-defined polysaccharide capsule. In *Methylocystis parvus* strain OBBP (= ATCC 35066) the capsule is

made up of a viscous exopolysaccharide polymer consisting mainly of D-glucose and L-rhamnose (Hou et al., 1978). *Methylocystis echinoides* strains possess characteristic pericellular prosthecate appendages called spinae (Coombs et al., 1976; Easterbrook, 1989), which have a cylindrical shape, about 300 nm long and 40 nm in diameter, and up to 300 are found on each cell (Suzina and Fikhte, 1977). The rib spacing on the individual spinae has been estimated at 6 to 12 nm (Easterbrook, 1989). The function of the spinae is currently unknown. On NMS agar, *Methylocystis parvus* forms pale pink colonies with a circular shape, convex elevation, smooth creamy consistency, and an entire edge. By comparison *Methylocystis echinoides* grows poorly on agar-solidified media, only producing scant growth and forming white pinpoint colonies, and thus this species should be routinely cultured in NMS liquid.

Methylocystis strains are strict aerobic, obligate methanotrophs that can only use methane and methanol as sole carbon and energy sources and have generation times estimated to be about 4–5 hours when growing in methane (Whittenbury et al., 1970b; Takeda, 1988). *Methylocystis* strains can form catalase, cytochrome c oxidase, and can fix nitrogen using an oxygen-sensitive nitrogenase (Murrell and Dalton, 1983b; Takeda, 1988). *Methylocystis* strains are mesophilic and neutrophilic, with best growth obtained at 25–35°C and at pH ranges of 6.5–7.5. Strains will grow at 10–40°C, with poor to moderate growth occurring at 37°C, and at pH 6.0–9.0. All *Methylocystis* strains are non-halophilic and completely inhibited by 2–3% (w/v) NaCl.

The mol% G+C of the DNA of *Methylocystis parvus* ranges from 63 to 67 (T_m), while the values for *Methylocystis echinoides* are slightly lower, ranging from 61 to 62 (T_m).

Methylocystis species possess a fatty acid profile consisting of mainly 18:1ω8c, 18:1ω7c and 18:0, with smaller amounts of cyclopropane fatty acids (Table 3). The profile is very similar to that of *Methylosinus*, differing only in the greater abundance of 18:0 and lower level of 16:1ω7c. The fatty acid 18:1ω8c is an unusual feature found almost exclusively in the Methylocystaceae, as it is very rarely encountered in other prokaryotes. The major lipopolysaccharide-derived hydroxy fatty acids include 3-OH 14:0 and 3-OH 18:0. In addition, *Methylocystis* species also possess unusual ω-1 hydroxy fatty acids with carbon chain lengths of 26 and 28 (Skerratt et al., 1992) making up about 15% of the total hydroxy fatty acids. These hydroxy acids have been previously detected in freshwater sediments (Mendoza et al., 1987), and *Methylocystis* and *Methylosinus* represent the first recognized biological sources of these particular lipids. *Methylocystis* species contain a suite of polar lipids, which vary slightly between strains due possibly to cultivation conditions (Makula, 1978; Andreev and Galchenko, 1983; Andreev and Galchenko, 1983). Polar lipids are present and include: phosphatidylethanolamine, phosphatidylglycerol, diphosphatidylglycerol, phosphatidylmethylethanol-amine, phosphatidyl-N,N-dimethylethanolamine and lysophosphatidylglycerol. The major quinone (Collins and Green, 1985) of *Methylocystis* species is Q-8.

Genus Methylosinus Strains of *Methylosinus trichosporium* have a pear-shaped to rod-like morphology with a width of 0.5–1.5 µm and a length of 1.5–3 µm. By comparison, *Methylosinus sporium* strains are of similar size but have a vibrioidal morphology. When grown in liquid media, both species form rosettes consisting of 4–6 cells. Several photomicrographs of the morphology of *Methylosinus* species have been published by Whittenbury et al. (1970a, 1970b). In logarithmic phase, cells divide by standard binary division; however, when cultures enter the stationary growth phase, an increasing proportion of cells reproduce by budding-off exospores as either rosettes or individual cells. Once sporulated, the mother cell ceases to divide or bud, becomes granulated and eventually lyses. Sporulating cells produce an extensive capsule compared to vegetative cells (Whittenbury et al., 1970a) (see Physiology section for more details on exospores). *Methylosinus* strains produce poly-β-hydroxybutyrate as an internal carbon reserve (Weaver et al., 1975; Best and Higgins, 1981) and have standard Gram-negative cell walls. However unlike most other Gram-negative bacteria, *Methylosinus* strains are very resistant to lysis by detergents (such as sodium dodecyl sulfate) and to lytic bacteria (Starostina and Pashkova, 1993). Lysozyme pretreatment (1 mg/ml at 37°C, for 30–60 min) followed by addition of 2% sodium dodecyl sulfate can force cell lysis. Colonies on NMS agar are nonpigmented, circular, and convex and have a smooth creamy texture and even entire edge. Some strains of *Methylosinus sporium* may form a brilliant-red prodigiosin-like pigment.

Methylosinus strains are strictly aerobic and obligately methanotrophic, and the only carbon and energy sources supporting growth are methane and methanol. All strains produce catalase and cytochrome c oxidase. Nitrate and ammonia salts, amino acids, yeast extract and casamino acids can be used as sources of nitrogen (Warner et al., 1983; Toukdarian and Lidstrom, 1984b). In addition, *Methylosinus* spp. are capable of fixing atmospheric nitrogen using an oxygen-sensitive nitrogenase (Murrell and Dalton, 1983b) which appears to be similar to the *Methylocystis* spp. enzyme. *Methylosinus* strains are mesophilic and

neutrophilic and grow at 10–40°C and pH 5.5–9.0. Optimal growth occurs at about 30°C and at pH 6.5–7.0. Most strains will not grow or grow poorly in presence of more than 0.3M NaCl and no strains have been found to require growth factors.

The DNA of *Methylosinus* strains have mol% G+C values ranging from 62 to 67 (T_m). *Methylosinus trichosporium* strains possess values of 62–63 (T_m), while *M. sporium* strains have values of 65–67 (T_m). *Methylosinus* species possess phospholipid fatty acids consisting mainly of 18:1ω8c, 18:1ω7c and 16:1ω7c with smaller amounts of 18:0 and cyclopropane fatty acids. The profile is very similar to that of *Methylocystis* but differs only by having more 16:1ω7c and less 18:0. The major lipopolysaccharide-derived hydroxy fatty acids include 3-OH 14:0, 3-OH 16:0 and 3-OH 18:0 (Bowman et al., 1991a). In addition, about 15% of the total hydroxy fatty acids of *Methylosinus sporium* are unusual ω-1 hydroxy fatty acids with carbon chain lengths of 26 and 28 (Skerratt et al., 1992). The neutral sugar components of the LPS oligosaccharide core in *Methylosinus trichosporium* OB3b include mostly rhamnose, glucose and heptose (Sutherland and Kennedy, 1986). *Methylosinus* spp. contain a suite of phospholipids (Makula, 1978; Andreev and Galchenko, 1983) including phosphatidyldimethylethanolamine, phosphatidylglycerol, phosphatidylmethylethanolamine, phosphatidylcholine and lysophosphatidylglycerol. The major quinone of *Methylosinus* is Q-8 (Collins and Green, 1985).

Preservation

Viability of most methanotrophs on plates or in liquid cultures is lost in a week or so, when they are deprived of methane. This is particularly true for cystless methanotrophs such as species of *Methylomicrobium* and *Methylosphaera* and also *Methylomonas* spp., for unknown reasons. However when placed under a 1 : 1 methane : air atmosphere plate or slant cultures, methanotrophs can be stored for several months at 4°C. Takeda (1988) found the shelf-life of type II methanotrophs was enhanced to over 12 months if cultures were kept in a 100% nitrogen atmosphere. *Methylocystis* and *Methylosinus* strains are amenable to cryopreservation (using 20% v/v dimethylsulfoxide or 20–30% v/v glycerol as a cryoprotectant) and to freeze drying (using 20% w/v skim milk or 10% v/v horse serum as cryoprotectant). Most Type I methanotrophs unfortunately do not survive freezing well (Nesterov et al., 1986). Cryopreservation with 20–30% glycerol may be used in most cases; however, vials must be replaced quite regularly (once every 1–2 months), which limits this method's usefulness. Type I methanotrophs can be kept more successfully in liquid nitrogen; however, recoverability usually becomes problematic after 6 months.

Physiology and Genetics

Detailed aspects of the genetics of methanotrophs as well as the biochemistry of the oxidation of methanol (and other C1 compounds) are covered in the chapter "Aerobic Methylotrophic Prokaryotes" in Volume 2. This section details the major physiological characteristics of obligate methanotrophs.

METABOLISM Methanotrophs possess a strictly aerobic metabolism, which uses C1 compounds. Most methanotrophs are limited to methane and methanol as substrates; however, a few species can utilize other C1 compounds including formate and methylamine. In some strains, complex carbon sources can be used as a source of carbon by methanotrophs grown in the presence of methane. In some cases this results in significant growth stimulation (Whittenbury and Dalton, 1981).

Methanotrophic strains can grow over a wide range of oxygen concentrations (<0.5% to 60% v/v) and are not microaerophilic, as has been suggested in some previous studies. Only when oxygen levels drop below 0.5% does growth become limiting (Ren et al., 1997) and in some species, significant growth still takes place at <0.1% oxygen. The work of Takeda (1988) and more recent research (Roslev and King, 1994; Roslev and King, 1995) have shown methanotrophs can survive anoxia for several months (presumably due to resting cell formation) and can rapidly respond when methane and oxygen once again become available.

RESTING STAGES Cyst Formation within the Methylococcaceae. Single- or multibodied spherical cysts, similar to those in *Azotobacter* species, develop in stationary growth phase cultures of *Methylobacter* and *Methylobacter*-like species (Whittenbury et al., 1970a; Malashenko et al., 1975b; Hazeu et al., 1980). Increased cyst formation is usually associated with increasing cell refractility and increased (tan) pigmentation of colonies. The cysts appear to confer some advantages to cells experiencing deprived conditions (such as lack of methane availability and drying), and thus the cysts are able to survive desiccation for several weeks. However the cysts do not confer heat resistance such as that provided by the exospores of *Methylosinus* species. In other Type I methanotrophs (*Methylomonas*, *Methylococcus* and *Methylocaldum* species), cyst formation is not as profuse as has been found in species of *Methylobacter* nor are cysts (appearing as single bodies) as well defined or as resistant to

dessication. Moreover, cysts contain glucan-type polysaccharides, which may act as an endogenous source of energy (Sutherland and MacKenzie, 1977). Cysts can be visualized by light microscopy by using a stain developed for Azotobacter- type cysts (Vela and Wyss, 1964).

Lipid Cysts of *Methylocystis.* In stationary growth phase, a proportion of *Methylocystis parvus* cells (but not its sister species *Methylocystis echinoides*) becomes refractile and more coccoid, eventually forming lipoidal cysts. These cysts are different from glucan-rich Type I methanotroph cysts by containing mostly poly-β-hydroxybutyrate and also possess a degree of desiccation resistance but no significant heat resistance.

Exospores of *Methylosinus.* As cultures enter the stationary growth phase, an increasing proportion of cells reproduce by budding-off exospore resting stages either when the cells are arranged in rosettes or by individual cells. When spores appear they are initially nonrefractile, Gram-negative, coccoidal bodies at the end of sporulating cells and as spores mature, they become increasing refractile and acid-fast. The malachite green spore stain (Doetsch, 1981) can be used to visualize mature spores. Thin sections show that exospores consist of an electron-dense outer coat (exosporium) surrounded by a cell wall derived from the parent cell. *Methylosinus trichosporium* exospores possess a capsular coat, attached to the exosporium but distinct from the parent cell's capsular layer (Reed et al., 1980). Within the exospore, there is a laminated inner coat and a poorly defined cortex (Reed et al., 1980; Dugan et al., 1982) lacking dipicolinic acid (a chemical present in the spores of Gram-positive bacteria) and no detectable respiratory activity. Exospores are resistant to desiccation, surviving 18 months of drying, and can also withstand heating at 85°C for 10 min and 10 min of ultrasonication.

Intracytoplasmic Membranes Methane oxidation usually takes place in methanotrophs within membrane systems referred to as intracytoplasmic membranes (ICM), which appear as a series of intracellular elaborate membrane folds that can be readily observed by electron microscopy. Intracytoplasmic membranes occur in two major ultrastructural forms. In Type I methanotrophs, ICM appear as a series of laminations or vesicular arrangements of the cytoplasmic membrane crossing the cell horizontally. In Type II methanotrophs, the ICM occur along the periphery of the cell wall and encloses a distinct lumen. Intracytoplasmic membranes are formed best when methanotrophs grow on methane, but ICM form to a lesser extent when methanotrophs grow on methanol (Best and Higgins, 1981). The amount of ICM increases in proportion to methane oxidation rates, acting to increase available surface area for the oxidation of methane. The affinity of methanotrophs to methane appears to vary with growth conditions, changing by greater than an order of magnitude (K_m 0.05→1 μM). The shifts in affinity are linked to the concentration of MMO within cells and changes in the relative levels of ICM (Dunfield et al., 1999). Methanotrophs in natural habitats experiencing low fluxes of methane are thus able to cope by maximizing methane-oxidizing efficiency. Methanotrophs experiencing oxygen limitation exhibit a reduction in ICM (Scott et al., 1981), while ICM synthesis is inhibited by removing copper from the medium (Prior and Dalton, 1985), which is linked to the concomitant repression in the synthesis of pMMO (see below).

Dissimilatory Methane Oxidation This pathway results in the oxidation of methane to CO_2 and is used by methanotrophs to generate energy and obtain carbon for biosynthesis. The first step of the pathway involves the oxidation of methane to methanol. Methanol is then oxidized to formaldehyde, the compound through which methanotrophs obtain the majority of their cellular carbon. A proportion of the formaldehyde is oxidized to formate and then to CO_2. In these latter steps, reducing power in the form of $NAD(P)H_2$ is generated and is used by methanotrophs to power electron transport and ATP synthesis. Only the first step of this pathway is dealt with in detail in this section, as the oxidation of methane to methanol is central to the growth of methanotrophs. The biochemistry and genetics of the remaining steps of the pathways are described in the chapter "Aerobic Methylotrophic Prokaryotes" in Volume 2.

Methane Monooxygenase (E.C.1.14.13.25) Methane monooxygenase (MMO) is the enzyme responsible for the oxidation of methane to methanol. It does this by incorporating a single atom of oxygen and the reaction has the following stoichiometry (Dworkin and Foster, 1956):

$$CH_4 + NAD(P)H_2 + O_2 \rightarrow CH_3OH + NAD(P^+) + H_2O$$

Particulate Methane Monooxygenase (pMMO). Two types of MMO have been found in methanotrophs; however, all methanotrophs possess pMMO—a copper-containing enzyme which is tightly bound within the ICM. Particulate MMO has an active site which includes copper and in the presence of copper limitation, pMMO synthesis is repressed, resulting in reduced growth yields on methane (unless the methanotroph possesses sMMO) and in

reduced ICM development. Previously it was found that the *amoA* gene, which codes for the active subunit of ammonia monooxygenase, hybridizes to methanotroph DNA (Semrau et al., 1995). Using *amoA* as a probe, the genes for pMMO were isolated and sequenced (Semrau et al., 1995) in three methanotrophs (*Methylococcus capsulatus*, *Methylomicrobium album* and *Methylosinus trichosporium)*. In each of these species pMMO contains two subunits, coded by *pmoA* and *pmoB* (45- and 27-kDa, respectively), which are present in duplicate gene copies in methanotrophs. The *pmoA* gene product has considerable amino acid sequence similarity to *amoA* product and the genes are believed evolutionarily related (Holmes et al., 1995a).

Soluble Methane Monooxygenase (sMMO) Some methanotrophs can form a soluble, membrane-free, form of MMO, sMMO. Soluble MMO is distributed irregularly amongst the methanotrophs and has been detected so far in most *Methylosinus* strains (Bowman et al., 1993a), some strains of *Methylocystis* (McDonald et al., 1997), *Methylococcus capsulatus* (i.e., strain ATCC 33009; Brusseau et al., 1990), and *Methylomonas methanica* (Koh et al., 1993). This enzyme has engendered considerable research interest owing to its enormous lack of substrate specificity. More than 250 known compounds of many different structural types (ranging from alkanes to heterocyclic compounds) can be oxidized by sMMO. This versatility has suggested a potential role for sMMO in bioremediation and industrial applications (see Applications section). Soluble MMO consists of three proteins including a non-heme hydroxylase, a ferredoxin-like reductase, and a regulatory coupling enzyme, with the separate proteins coded by a single gene cluster. The *mmo* operon has been characterized and sequenced in both *Methylococcus capsulatus* (Bath) (Stainthorpe et al., 1990) and *Methylosinus trichosporium* (OB3b) (Cardy et al., 1991). The hydroxylase component is a dimer of three separate subunits and has a non-heme di-iron active site (Nordlund et al., 1992). It is the unusual nature of this active site that is responsible for the potent oxidative nature of sMMO. The individual subunits are coded by *mmoX*, *mmoY* and *mmoZ*, respectively. The 3-dimensional structure of the non-heme hydroxylase enzyme has been resolved (Rosenzweig et al., 1993). The ferrodoxin-like reductase enzyme (coded by *mmoA* and *mmoC*) transfers electrons to the hydroxylase for the catalysis of methane oxidation. The coupling protein, coded by *mmoB*, links the reductase and the hydroxylase. It is thought this enzyme has a regulatory role, decoupling the hydroxylase and reductase possibly when formaldehyde reaches a critical level. Soluble MMO synthesis in methanotrophs is repressed in the presence of copper (at levels as low as 50 nM) with concurrent increased synthesis of pMMO, and thus it is thought sMMO may have evolved in conditions of copper limitation (Hanson and Hanson, 1996). Limitation in copper may arise from chelation, adsorption and complexation processes, especially with various organic compounds. Copper-limited environments such as groundwater are often dominated by sMMO-producing methanotrophs (Bowman et al., 1993a). When growing methanotrophs for the purpose of making sMMO, all glassware and media should be free of trace copper ions. Soluble MMO can be readily detected by the naphthalene oxidation assay (Brusseau et al., 1990; Graham et al., 1992), by gene probe (for example using regulatory gene, mmoB; Stainthorpe et al., 1991), or by PCR using specific primers (McDonald et al., 1995; Murrell et al., 1998). The genes for both pMMO and sMMO are genetically distinct (Martin and Murrell, 1995); however, they have a common copper-inducible regulatory pathway (Neilsen et al., 1997).

Carbon Assimilation Pathways Methanotrophs fix carbon in the form of formaldehyde, which is rapidly cycled owing to its high toxicity. Formaldehyde is fixed by two different pathways in methanotrophs: the ribulose monophosphate (RuMP) pathway used by Type I methanotrophs and the serine pathway used by Type II methanotrophs (Anthony, 1982; Hanson and Hanson, 1996). By assaying the key enzymes of these pathways, Type I and II methanotrophs may be distinguished. In the case of the RuMP pathway, the key enzyme is hexulose phosphate synthase, whereas in the serine pathway the key enzyme is hydroxypyruvate reductase. More details on these pathways can be found in the chapter "Aerobic Methylotrophic Prokaryotes" in Volume 2.

Nitrogen Metabolism Most methanotrophs assimilate ammonia and nitrate by the glutamine synthetase-glutamine 2-oxoglutarate aminotransferase system (Shishkina and Trotsenko, 1979; Murrell and Dalton, 1983a; Toukdarian and Lidstrom, 1984b). Methanotrophs also can assimilate nitrogen from amino acids and other complex mixtures such as yeast extract. In addition, many methanotrophs are able to fix atmospheric nitrogen and include all Type II methanotrophs and various members of the Type I methanotrophs, including *Methylomonas* spp., *Methylococcus capsulatus*, and *Methylosphaera hansonii*. In most cases the nitrogenase formed is oxygen-sensitive (Murrell and Dalton, 1983b;

Takeda, 1988; Zhivotchenko et al., 1995), except for *Methylosphaera hansonii*, which appears to possess a more oxygen-tolerant enzyme (Bowman et al., 1997). In the case of methanotrophs with oxygen-sensitive nitrogenase, reducing the oxygen partial pressure in the headspace enhances growth on agar under nitrogen-free conditions; however, oxygen concentrations over 10% will abolish nitrogenase activity almost completely (Zhivotchenko et al., 1995). A number of homologs of *nifH* have been shown in strains such as *Methylomonas methanica*, which appeared unable to fix nitrogen in vitro (Oakley and Murrell, 1988). Southern blotting indicates the *nif* genes of various methanotrophs are homologous with each other and genes of *Klebsiella pneumoniae* (Toukdarian and Lidstrom, 1984a; Oakley and Murrell, 1988).

Applications

Methanotrophs have been considered as cheap sources of single-cell protein (Anthony, 1982) and poly-beta-hydroxybutyrate (a natural polymer; Hou, 1984; Wendlandt et al., 1998). However most biotechnologically directed studies of methanotrophs have focused on the capability of sMMO from *Methylosinus trichosporium* OB3b to co-oxidize a wide range of carbon substrates. The compounds oxidized are too many to list but include a wide range of aliphatic, heterocyclic and aromatic compounds (Burrow et al., 1984). Several studies have focused on the industrial applications of this biocatalytic ability, in particular in the production of epoxides for plastics manufacture (Hou, 1984), but the primary focus of research has been in the bioremediation field, and this aspect has been reviewed extensively (Sullivan et al., 1998). *Methylosinus trichosporium* OB3b and other strains forming sMMO can co-metabolize a range of chlorinated aliphatic compounds including major groundwater pollutants such as trichloroethylene, chloroform and tetrachloroethylene (Oldenhuis et al., 1989; Tsien et al., 1989; Castro et al., 1996; Hamamura et al., 1997; Moran and Hickey, 1997; Tartakovsky et al., 1998). However, the industrial application of methanotrophs has been hampered by their relatively slow growth and requirement for methane, a potentially explosive substrate which also competitively inhibits co-metabolic reactions. Another problem is that trace copper levels can suppress sMMO activity, thus eliminating or reducing transformation rates (Oldenhuis et al., 1989; Lontoh and Semrau, 1998). To overcome this, constitutive mutants lacking sMMO activity suppression [RG7] have been created by chemical mutagenesis and by marker exchange (Phelps et al., 1992; Fitch et al., 1993; Martin and Murrell, 1995; Tellez et al., 1998). These mutants have been shown to be able to co-metabolize trichloroethylene in the presence of high levels of copper. Some of these copper-tolerant mutants have been utilized in treatments of chlorinated aliphatic pollutants (Tschantz et al., 1995). In addition, the sMMO gene cluster has been successfully cloned into *Pseudomonas putida* F1, which is not only able to degrade trichloroethylene but can grow much faster and lacks the problem associated with methane competitive inhibition (Jahng and Wood, 1994; Jahng et al., 1996).

Literature Cited

Ambramochkina, F. N., L. V. Bezrukova, A. V. Koshelev, V. F. Galchenko, and M. V. Ivanov. 1987. Microbial oxidation of methane in a body of fresh water. Microbiologiya (English Translation) 56:375–382.

Andreev, L. V., and V. F. Galchenko. 1978. Fatty acid composition and identification of methanotrophic bacteria. Dokl. Akad. Nauk SSSR 269:1461–1468.

Andreev, L. V., and V. F. Galchenko. 1983. Phospholipid composition and differentiation of methanotrophic bacteria. J. Liquid Chromatogr. 6:2699–2707.

Anthony, C. 1982. The Biochemistry of Methylotrophs. Academic Press Ltd., London.

Bender, M., and R. Conrad. 1992. Kinetics of CH_4 oxidation in oxic soils, exposed to ambient air or high CH4 mixing ratios. FEMS Microbiol. Ecol. 101:261–270.

Bender, M., and R. Conrad. 1994. Methane oxidation activity in various soils and freshwater sediments: occurrence, characteristics, vertical profiles and distribution on grain size fractions. J. Geophys. Res. 99:16531–16540.

Bezrukova, L. V., Y. I. Nikolenklo, A. I. Nesterov, V. F. Galchenko, and M. V. Ivanov. 1983. Comparative serological analysis of methanotrophic bacteria. Microbiologiya (English Translation) 52:800–805.

Best, D. J., and I. J. Higgins. 1981. Methane-oxidizing activity and membrane morphology in a methanol grown obligate methanotroph, Methylosinus trichosporium OB3b. J. Gen. Microbiol. 125:73–84.

Bird, C. C., W. M. Lynch, F. J. Pirt, W. W. Reid, C. J. W. Brooks, and B. C. Middleditch. 1971. Steroids and squalene in Methylococcus capsulatus grown on methane. Nature 230:473–474.

Bodrossy, L., E. M. Holmes, A. J. Holmes, K. L. Kovacs, and J. C. Murrell. 1997. Analysis of 16S rRNA and methane monooxygenase gene sequences reveals a novel group of thermotolerant and thermophilic methanotrophs, Methylocaldum gen. nov. Arch. Microbiol. 168:493–503.

Boon, P. I., P. Virtue, and P. D. Nichols. 1996. Microbial consortia in wetlnd sediments—a biomarker analysis of the effects of hydrological regime, vegetation and season on benthic microbes. Mar. Fresh. Res. 47:27–41.

Boschker, H. T. S., S. C. Nold, P. Wellsbury, D. Bos, W. Degraaf, R. Pel, J. J. Parkes, and T. E. Cappenburg. 1998. Direct linking of microbial populations to specific biogeochemical processes by ^{13}C-labelling of biomarkers. Nature 392:801–805.

Bowman, J. P. 1992. The Systematics of Methane-Utilising Bacteria. University of Queensland. Brisbane, 140.

Bowman, J. P., L. Jimenez, I. Rosario, T. C. Hazen, and G. S. Sayler. 1993a. Characterization of the methanotrophic bacterial community present in a trichloroethylene-contaminated subsurface groundwater site. Appl. Environ. Microbiol. 59:2380–2387.

Bowman, J. P., S. A. McCammon, and J. H. Skerratt. 1997. Methylosphaera hansonii gen. nov. sp. nov. a psychrophilic, group I methanotroph from Antarctic marine-salinity, meromictic lakes. Microbiology 143:1451–1459.

Bowman, J. P., J. H. Skerratt, P. D. Nichols, and L. I. Sly. 1991a. Phospholipid fatty acid and lipopolysaccharide fatty acid signature lipids in methane-utilising bacteria. FEMS Microbiol. Ecol. 85:15–22.

Bowman, J. P., L. I. Sly, and A. C. Hayward. 1990. Methylomonas fodinarum sp. nov. and Methylomonas aurantiaca sp. nov.: two closely related type I obligate methanotrophs. Syst. Appl. Microbiol. 13:279–287.

Bowman, J. P., L. I. Sly, and A. C. Hayward. 1991b. Contribution of genome characteristics to the assessment of taxonomy of obligate methanotrophs. Int. J. Syst. Bacteriol 41:301–305.

Bowman, J. P., L. I. Sly, P. D. Nichols, and A. C. Hayward. 1993b. Revised taxonomy of the methanotrophs: description of Methylobacter gen. nov., emendation of Methylococcus, validation of Methylosinus and Methylocystis species, and a proposal that the family Methylococcaceae includes only the group I methanotrophs. Int. J. Syst. Bacteriol. 43:735–753.

Bowman, J. P., L. I. Sly, and E. Stackebrandt. 1995. The phylogenetic position of the family Methylococcaceae. Int. J. Syst. Bacteriol. 45:182–185.

Bratina, B. J., G. A. Brusseau, and R. S. Hanson. 1992. Use of 16S rRNA analysis to investigate phylogeny of methylotrophic bacteria. Int. J. Syst. Bacteriol. 42:645–648.

Brigmon, R. L., M. M. Franck, J. S. Bray, D. F. Scott, K. D. Lanclos, and C. B. Fliermans. 1998. Direct immunofluoresence and enzyme-linked immunosorbent assays for evaluating organic contaminant degrading bacteria. J. Microbiol. Methods 32:1–10.

Brown, L. R., and R. J. Strawinski. 1958. Intermediates in the oxidation of methane. Bacteriol. Proc. 58:96–132.

Brusseau, G. A., E. Bulygina, and R. S. Hanson. 1994. Phylogenetic analysis and development of probes differentiating methylotrophic bacteria. Appl. Environ. Microbiol. 60:626–636.

Brusseau, G. A., H. C. Tsien, R. S. Hanson, and I. P. Wackett. 1990. Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase. Biodegradation 1:19–29.

Burrow, K. J., A. Cornish, D. Scott, and I. J. Higgins. 1984. Substrate specificities of the soluble and particulate methane monooxygenases of Methylosinus trichosporium OB3b. J. Gen. Microbiol. 130:3327–3333.

Calhoun, A., and G. M. King. 1998. Charcaterization of root-associated methanotrophs from three freshwater macrophyes—Pontederia cordata, Sparganium eurycarpum, and Sagittaria latifolia. Appl. Environ. Microbiol. 64:1099–1105.

Cardy, D. L. N., V. Laidler, G. P. C. Salmond, and J. C. Murrell. 1956. The methane monooxygenase gene cluster of Methylosinus trichosporium: cloning and sequencing of the mmoC gene. Arch. Microbiol. 1991: 477–483.

Castro, C. E., S. K. O'Shea, W. Wang, and E. W. Bartnicki. 1996. Biohalogenation—oxidative and hydrolytic pathways in the transformations of acetonitrile, chloroacetonitrile, chloroacetic acid and chloroacetamide by Methylosinus trichosporium OB3b. Environ. Sci. Technol. 30:1180–1184.

Cavanaugh, C. M. 1993. Methanotrop-invertebrate symbioses in the marine environment: ultrastructural, biochemical and molecular studies. J. C. Murrell and D. P. Kelly, Microbial Growth on C1 Compounds. Intercept Press. Andover, 315-328.

Cavanaugh, C. M., P. R. Levering, J. S. Maki, R. Mitchell, and M. E. Lidstrom. 1987. Symbiosis of methylotrophic bacteria and deep-sea mussels. Nature 325:346–348.

Childress, J. J., C. R. Fisher, J. M. Brooks, M. C. Kennicut, R. Bidigare, and A. E. Anderson. 1986. A methanotrophic marine molluscan (Bivalvia: Mytilidae) symbiosis: mussels fueled by gas. Science 233:1306–1308.

Collins, M. D., and P. N. Green. 1985. Isolation and characterization of a novel coenzyme Q from some methane-oxidizing bacteria. Biochem. Biophys. Res. Com. 133:1125–1131.

Conrad, R., P. Frenzel, and Y. Cohen. 1995. Methane emission from hypersaline microbial mats: lack of aerobic methane oxidation activity. FEMS Microbiol. Ecol. 610:1–9.

Coombs, R. W., J. A. Verpoorte, and K. B. Easterbrook. 1976. Protein conformation in bacterial spinae. Biopolymers 15:2353–2369.

Davis, J. B., V. G. Coty, and J. P. Stanley. 1964. Atmospheric nitrogen fixation by methane-oxidizing bacteria. J. Bacteriol. 88:468–472.

Dedysh, S. N., N. S. Panikov, and J. M. Tiedje. 1998. Acidophilic methanotrophic communities from sphagnum peat bogs. Appl. Environ. Microbiol. 64:922–929.

Distel, D. L., and C. M. Cavanaugh. 1994. Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbionts in marine bivalves. J. Bacteriol. 176:1932–1938.

Doetsch, R. N. 1981. Determinative methods of light microscopy. P. GerhardtManual of Methods for General Bacteriology. American Society for Microbiology. Washington, DC, 21–33.

Dugan, P., J. Titus, W. M. Reed, and R. M. Pfister. 1982. Exospore formation in Methylosinus trichosporium. J. Bacteriol. 149:354–360.

Dunfield, P. F., W. Liesack, T. Henckel, R. Knowles, and R. Conrad. 1999. High affinity methane oxidation by a soil enrichment culture containing a Type II methanotroph. Appl. Environ. Microbiol. 65:1009–1014.

Easterbrook, K. B. 1989. Spinate Bacteria. J. T. Staley, M. P. Bryant, N. Pfennig and J. G. Holt, Bergey's Manual of Systematic Bacteriology. The Williams and Wilkins Co. Baltimore, 1991-1993.

Edwards, C., B. A. Hales, G. H. Hall, I. R. McDonald, J. C. Murrell, R. Pickup, D. A. Ritchie, J. R. Saunders, B. M. Simon, and M. Upton. 1998. Microbiological processes in the terrestrial carbon cycle - methane cycling in peat. Atmosph. Environ. 32:3247–3255.

Escoffier, S., J. Lemer, and P. A. Roger. 1997. Enumeration of methanotrophic bacteria in ricefield soils by plating and MPN techniques—a critical approach. Eur. J. Soil Biol. 33:41–51.

Faust, U., P. Prave, and D. A. Sukatsch. 1977. Continuous biomass productionfrom methanol by Methylomonas clara. J. Ferment. Technol. 55:609–614.

Fitch, M. W., D. W. Graham, R. G. Arnold, S. K. Agarwal, P. Phelps, G. E. Speitel, and G. Georgiou. 1993. Phenotypic

characterization of copper-resistant mutants of Methylosinus trichosporium OB3b. Appl. Environ. Microbiol. 59:2771–2776.

Foster, J. W., and R. H. Davis. 1966. A methane-dependent coccus, with notes on classification of obligate methane-utilizing bacteria. J. Bacteriol. 91:1924–1931.

Franzmann, P. D., Y. T. Liu, D. L. Balkwill, H. C. Aldrich, E. C. DeMacario, and D. R. Boone. 1997. Methanogenium frigidum sp. nov., a psychrophilic, H^2-using methanogen from Ace Lake, Antarctica. Int. J. Syst. Bacteriol. 47:1068–1072.

Galchenko, V. F. 1977. New species of Methylocystis. Y. R. MalashenkoProceedings of the 2nd Symposium on the Growth of Microorganisms. The Academy of Sciences, USSR. Moscow, 1–17.

Galchenko, V. F. 1994. Sulfate reduction, methane production and methane oxidation in various waterbodies of the Bunger Hills Osasis of Antarctica. Microbiologiya (English Translation) 63:388–396.

Galchenko, V. F., F. N. Ambramochkina, L. V. Bezrukova, E. N. Sokolova, and M. V. Ivanov. 1988. Species composition of aerobic methanotrophic microflora in the Black Sea. Microbiologiya (English Translation) 57:248–253.

Galchenko, V. F., and A. I. Nesterov. 1981. Numerical analysis of protein electrophoretograms of obligate methane-utilizing bacteria. Microbiologiya (English Translation) 50:725–730.

Galchenko, V. F., V. N. Shishkina, V. S. Tyurin, and Y. A. Trotsenko. 1975. Isolation of pure cultures of methanotrophs and their properties. Microbiologiya (English Translation) 50:725–730.

Galchenko, V. F., V. N. Shishkina, N. E. Suzina, and Y. A. Trotsenko. 1977. Isolation and properties of new strains of obligate methanotrophs. Microbiologiya (English Translation) 46:723–728.

Graham, D. W., D. G. Korich, R. P. LeBlanc, N. A. Sinclair, and R. G. Arnold. 1992. Applications of a colorimetric plate assay for soluble methane monooxygenase activity. Appl. Environ. Microbiol. 58:2231–2236.

Guckert, J. B., D. B. Ringelberg, D. C. White, R. S. Hanson, and B. J. Bratina. 1991. Membrane fatty acids as phenotypic markers for the polyphasic approach to taxonomy of methylotrophs within the Proteobacteria. J. Gen. Microbiol. 137:2631–2641.

Guezennec, J. andA. Fiali-Medioni. 1996. Bacterial abundance and diversity in the Barbados Trench determined by phospholipid analysis. FEMS Microbiol. Ecol. 19:83–93.

Hamamura, N., C. Page, T. Long, L. Semprini, and D. J. Arp. 1997. Chloroform cometabolism by butane-grown CF8, Pseudomonas butanovora, Mycobacterium vaccae job5 and methane-grown Methylosinus trichosporium OB3b. Appl. Environ. Microbiol 63:3607–3613.

Hanson, R. S., B. J. Bratina, and G. A. Brusseau. 1993. Phylogeny and ecology of methylotrophic bacteria. J. C. Murrell and D. P. Kelley, Microbial Growth on C1 Compounds. Intercept Press. Andover, 285–302.

Hanson, R. S., and T. E. Hanson. 1996. Methanotrophic bacteria. Microbiol. Rev. 60:439–471.

Hazeu, W., W. H. Batenburg-van der Vegte, and J. C. de Bruyn. 1980. Some characteristics of Methylococcus mobilis sp. nov. Arch. Microbiol. 124:211–220.

Hazeu, W., and P. J. Steenis. 1970. Isolation and characterization of two vibrio-shaped methane-oxidizing bacteria. Antonie van Leeuwenhoek 36:67–72.

Haubold, R. 1978. Two different types of surface structures of methane-utilizing bacteria. J. Basic Microbiol. 18:511–515.

Holmes, A. J., A. Costello, M. E. Lidstrom, and J. C. Murrell. 1995a. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol. Lett. 132:203–208.

Holmes, A. J., N. J. P. Owens, and J. C. Murrell. 1996. Detection of novel marine methanotrophs using phylogenetic and functional gene probes afetr methane enrichment. Microbiology 141:1947–1955.

Holmes, A. J., N. P. J. Owens, and J. C. Murrell. 1996. Molecular analysis of enrichment cultures of marine methane-oxidising bacteria. J. Exp. Mar. Biol. Ecol. 203:27–38.

Hou, C. T. 1984. Methylotrophs: Microbiology, Biochemistry and Genetics. CRC Press, Boca Raton.

Hou, C. T., A. I. Laskin, and R. N. Patel. 1978. Growth and polysaccharide production by Methylcystis parvus OBBP on methanol. Appl. Environ. Microbiol. 37:800–803.

Jahnke, L. L., and P. D. Nichols. 1986. Methyl sterol and cyclopropane fatty acid composition of Methylococcus capsulatus grown at low oxygen tensions. J. Bacteriol. 167:238–242.

Jahnke, L. L., H. Stan-Lotter, K. Kato, and L. I. Hochstein. 1992. Presence of methyl sterol and bacteriohopanepolyol in an outer-membrane preparation from Methylococcus capsulatus (Bath). J. Gen. Microbiol. 138:1759–1766.

Jahng, D. J., and T. K. Wood. 1994. Trichloroethylene and chloroform degradtion by a recombinant pseudomonad expressing soluble methane monooxygenase from Methylosinus trichosporium OB3b. Appl. Environ. Microbiol. 60:2473–2482.

Jahng, D. J., C. S. Kim, R. S. Hanson, and T. K. Wood. 1996. Optimization of trichloroethylene degradation using soluble methane monooxygenase of Methylosinus trichosporium OB3b expressed in recombinant bacteria. Biotechnol. Bioeng. 51:349–359.

Jenkins, O., D. Byrom, and D. Jones. 1987. Methylophilus: a new genus of methanol-utilizing bacteria. Int. J. Syst. Bacteriol. 37:446–458.

Jensen, S, L. Ovreas, F. L. Daae, and V. Torsvik. 1998. Diversity in methane enrichments from agricultural soil revealed by DGGE separation of PCR-amplified 16S rDNA fragments. FEMS Microbiol. Ecol. 26:17–26.

King, G. M. 1994. Methanotrophic associations with the roots and rhizomes of aquatic vegetation. Appl. Environ. Microbiol. 60:3220–3227.

Koh, S. C., J. P. Bowman, and G. S. Sayler. 1993. Soluble methane monooxygenase production and trichloroethylene degradation by a Type I methanotroph Methylomonas methanica 68-1. Appl. Environ. Microbiol. 59:960–967.

Kuono, K., T. Oki, H. Komura, and A. Ozaki. 1973. Isolation of new methanol-utilizing bacteria and its thiamine requirement for growth. J. Gen. Appl. Microbiol. 19:11–21.

Kussmaul, M., M. Wilimzig, and E. Bock. 1998. Methanotrophs and methanogens in masonry. Appl. Environ. Microbiol. 64:4350–4352.

Lees, V. N., J. P. Owens, and J. C. Murrell. 1991. Nitrogen metabolism of marine methanotrophs. Arch. Microbiol. 157:60–63.

Lidstrom, M. E. 1988. Isolation and characterization of marine methanotrophs. Antonie van Leeuwenhoek 54:189–199.

Lontoh, S., and J. D. Semrau. 1998. Methane and trichloroethylene degradation by Methylosinus trichosporium OB3b expressing particulate methane monooxygenase. Appl. Environ. Microbiol. 64:1106–1114.

Makula, R. A. 1978. Phospholipid composition of methane-utilizing bacteria. J. Bacteriol. 134:771–777.

Malashenko, Y. R., Y. Kaier, E. N. Budkova, Y. Isagulova, U. Berger, T. P. Krishtab, D. V. Chernyshenko, and V. A. Romanovskaya. 1987. Methane-oxidizing microflora in bodies of fresh and salt water. Microbiologiya (English Translation) 56:115–120.

Malashenko, Y. R., V. A. Romanovskaya, and E. I. Kvashnikov. 1972. Taxonomy of bacteria utilizing gaseous hydrocarbons. Microbiologiya (English Translation) 41:777–783.

Malashenko, Y. R., V. A. Romanovskaya, and V. N. Bogachenko. 1975a. Thermophilic and thermtolerant methane-assimilating bacteria. Microbiologiya (English Translation) 44:638–643.

Malashenko, Y. R., V. A. Romanovskaya, V. N. Bogachenko, and A. D. Shved. 1975b. Thermophilic and thermotolerant methane-assimilating bacteria. Microbiologiya (English Translation) 44:855–862.

Martin, H., and J. C. Murrell. 1995. Methane monooxygenase mutants of Methylosinus trichosporium constructed by marker-exchange mutagenesis. FEMS Microbiol. Lett. 127:243–248.

McDonald, I. R., G. H. Hall, R. W. Pickup, and J. C. Murrell. 1996. Methane oxidation potential and preliminary analysis of methanotrophs in blanket peat bog using molecular ecology techniques. FEMS Microbiol. Ecol. 21:197–211.

McDonald, I. R., E. M. Kenna, and J. C. Murrell. 1995. Detection of methanotrophic bacsteria in environmental samples with PCR. Appl. Environ. Microbiol. 61:116–121.

McDonald, I. R., and J. C. Murrell. 1997a. The methanol dehydrogenase structural gene mxaF and its use as a functional gene probe for methanotrophs and methylotrophs. Appl. Environ. Microbiol. 63:3218–3224.

McDonald, I. R., and J. C. Murrell. 1997b. The particulate methane monooxygenase gene pmoA and its use as a functional gene probe for methanotrophs. FEMS Microbiol. Lett. 156:205–210.

McDonald, I. R., H. Uchiyama, S. Kambe, O. Yagi, and J. C. Murrell. 1997. The soluble methane monooxygenase gene cluster of the trichloroethylene-degrading methanotroph Methylocystis sp. M. Appl. Environ. Microbiol. 63:1898–1904.

Mendoza, Y. A., F. O. Gulacar, Z. L. Hu, and A. Buchs. 1987. Unsubstituted and hydroxy substituted acids in recent lacustrine sediment. Int. J. Environ. Anal. Chem. 31:107–127.

Meyer, J. 1977. New data on taxonomy of methane-utilizing bacteria. Y. R. Malashenko, Proceedings of the 2nd Symposium on the Growth of Microorganisms on C1 Compounds. Academy of Sciences, USSR. Moscow, 17–20.

Meyer, J., R. Haubold, J. Heyer, and W. Bockel. 1986. Contribution to the taxonomy of methanotrophic bacteria: correlation between membrane type and GC-value. Z. Allg. Mikrobiol. 26:155–160.

Moran, B. N., and W. J. Hickey. 1997. Trichloroethylene biodegradtion by mesophilic and psychrophilic ammonia oxidizers and methanotrophs in groundwater microcosms. Appl. Environ. Microbiol. 63:3866–3871.

Morinaga, Y., S. Yamanaka, S. Otsuka, and Y. Hirose. 1976. Characteristics of a newly isolated methane-utilizing bacterium, Methylomonas flagellata sp. nov. Agric. Biol. Chem. 40:1539–1545.

Murray, R. G. E., D. J. Brenner, R. R. Colwell, P. De Vos, M. Goodfellow, P. A. D. Grimont, N. Pfennig, E. Stackebrandt, and G. Zarvarzin. 1990. Report of the ad hoc committee on approaches to taxonomy within Proteobacteria. Int. J. Syst. Bacteriol. 40:213–215.

Murrell, J. C., and H. Dalton. 1983a. Ammonia assimilation in Methylococcus capsulatus (Bath) and other obligate methanotrophs. J. Gen. Microbiol. 120:1197–1206.

Murrell, J. C., and H. Dalton. 1983b. Nitrogen fixation in obligate methanotrophs. J. Gen. Microbiol. 129:3481–3486.

Murrell, J. C., I. R. McDonald, and D. G. Bourne. 1998. Molecular methods for the study of methanotroph ecology. FEMS Microbiol. Ecol. 27:103–114.

Neilsen, A. K., K. Gerdes, and J. C. Murrell. 1997. Copper-dependent reciprocal transcriptional regulation of methane monoxygenase genes in Methylococcus capsulatus and Methylosinus trichosporium. Mol. Microbiol. 25:399–409.

Nesterov, A. I., A. V. Koshelev, V. F. Galchenko, and M. V. Ivanov. 1986. Survival of obligate methanotrophous bacteria following lyophilization and subsequent storage. Microbiologiya (English Translation) 55:215–221.

Neunlist, S., and M. Rohmer. 1985. Novel hopanoids from the methylotrophic bacteria Methylococcus capsulatus and Methylomonas methanica: 22(S)-35-aminobacteriohopane-30,31,32,33,34-pentol and (22S)-35-aminomethylaminobacteriohopane-30,31,32,33,34-pentol. Biochem. J. 231:635–639.

Nichols, P. D., J. M. Henson, C. P. Antworth, J. Parsons, J. T. Wilson, and D. C. White. 1987. Detection of a microbial consortium including Type II methanotrophs by use of phospholipid fatty acids in aerobic halogenated-degrading soil columns enriched with natural gas. Environ. Toxicol. Chemi. 6:89–97.

Nichols, P. D., G. A. Smith, C. P. Antworth, R. S. Hanson, and D. C. White. 1985. Phospholipid and lipopolysaccharide normal and hydroxy fatty acids as potential signatures for methane-utilizing bacteria. FEMS Microbiol. Ecol. 32:327–335.

Nordlund, P., H. Dalton, and H. Eklund. 1992. The active site structure of methane monooxygenase is closely related to the binuclear iron center of ribonucleotide reductase. FEBS Lett. 307:257–262.

Oakley, C. J., and J. C. Murrell. 1988. nifH genes in the obligate methane oxidizing bacteria. FEMS Microbiol. Lett. 49:53–57.

Oldenhuis, R., R. L. J. M. Vink, D. B. Janssen, and B. Witholt. 1989. Degradation of chlorinated aliphatic hydrocarbons by Methylosinus trichosporium OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55:2819–2826.

Omelchenko, M. V., L. V. Vasileva, G. A. Zavarzin, N. D. Saveleva, A. M. Lysenko, L. L. Mityushina, V. N. Khmelenina, and Y. A. Trotsenko. 1996. A novel psychrophilic methanotroph of the genus Methylobacter. Microbologiya (English Translation) 65:339–343.

Oremland, R. S., and C. W. Cuthbertson. 1992. Importance of methane-oxidizing bacteria in the methane budget as

revealed by the use of a specific inhibitor. Nature 356:421–423.

Phelps, P. A., S. K. Agarwal, G. E. Speitel, and G. Georgiou. 1992. Methylosinus trichosporium OB3b mutants having constituitive expression of soluble methane monooxygenase in the presence of high levels of copper. Appl. Environ. Microbiol. 58:3701–3708.

Prior, S. D., and H. Dalton. 1985. The effect of copper ions on membrane content and methane monooxygenase activity in methanol-grown cells of Methylococcus capsulatus (Bath). J. Gen. Microbiol. 131:155–163.

Reeburgh, W. S., S. C. Whalen, and M. L. Alpern. 1993. The role of methylotroph in the global methane budget. J. C. Murrell and D. P. Kelley, Microbial Growth on C1 Compounds. Intercept Press. Andover, 1–14.

Reed, W. M., and P. R. Dugan. 1978. Distribution of Methylomonas methanica and Methylosinus trichosporium in Cleveland Harbor as determined by an indirect fluorescent antibody-membrane filter technique. Appl. environ. Microbiol. 35:422–430.

Reed, W. M., J. A. Titus, P. R. Dugan, and R. M. Pfister. 1980. Structure of Methylosinus trichosporium exospores. J. Bacteriol. 141:908–913.

Ren, T., J. A. Amaral, and R. Knowles. 1997. The response of methane consumption by pure cultures of methanotrophic bacteria to oxygen. Can. J. Microbiol. 43:925–928.

Romanovskaya, V. A., Y. R. Malashenko, and V. N. Bogachenko. 1978. Corrected diagnoses of the genera and species of methane-utilizing bacteria. Microbiologiya (English Translation) 47:96–103.

Rosenzweig, A. C., C. A. Frederick, S. J. Lippard, and P. Nordlund. 1993. Crystal structure of a bacterial nonhaem iron hydroxylase that catalyses the biological oxidation of methane. Nature 366:537–543.

Roslev, P., and G. M. King. 1994. Survival and recovery of methanotrophic bacteria starved under oxic and anoxic conditions. Appl. Environ. Microbiol. 60:2602–2608.

Roslev, P., and G. M. King. 1995. Aerobic and anaerobic starvation metabolism in methanotrophic bacteria. Appl. Environ. Microbiol. 61:1563–1570.

Ross, J. L., P. I. Boon, P. Ford, and B. T. Hart. 1997. Detection and quantification with 16S rRNA probes of planktonic methylotrophic bacteria ina floodplain lake. Microb. Ecol. 34:97–108.

Saralov, A. I., and T. R. Babnazarov. 1982. The microflora and molecular nitrogen fixation in takyr-like soils of rice fields in Karakalpacia. Microbiologiya (English Translation) 51:847–853.

Saralov, A. I., I. N. Krylova, E. E. Saralova, and S. I. Kuznetsov. 1984. Distribution and species composition of methane-oxidizing bacteria in lakewaters. Microbiologiya (English Translation) 53:695–700.

Schmalijohann, R., and H. J. Fluegel. 1987. Methane-oxidizing bacteria in pogonophora. Sarsia 72:91–98.

Scott, D., J. Brannan, and I. J. Higgins. 1981. The effect of growth conditions on intracytoplasmic membranes and methane monooxygenase activities in Methylosinus trichosporium OB3b. J. Gen. Microbiol. 125:63–72.

Sieburth, J. M., P. W. Johnson, V. M. Church, and D. C. Laux. 1993. C1 bacteria in the water column of Chesapeake Bay, USA. III. Immunologic relationships in the type species of marine monomethylamine- and methane-oxidizing bacteria to wild estuarine and oceanic cultures. Mar. Ecol. Prog. Ser. 95:91–102.

Sieburth, J. M., P. W. Johnson, M. A. Eberhardt, M. E. Sieracki, M. Lidstrom, and D. Laux. 1987. The first methane-oxidizing bacterium from the upper mixed layer of the deep ocean, Methylomonas pelagica sp. nov. Curr. Microbiol. 14:285–293.

Skerman, V. B. D., V. McGowan, and P. H. A. Sneath. 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225–420.

Skerratt, J. H., P. D. Nichols, J. P. Bowman, and L. I. Sly. 1992. Occurrence and significance of long-chain (w-1)-hydroxy fatty acids in methane-utilising bacteria. Org. Geochem. 18:92–99.

Southward, A. J., E. C. Southward, P. R. Dando, G. H. Rau, G. Felbeck, and H. Fluegel. 1981. Bacterial symbionts and low 13C/13C ratios in tissues of Pogonophora indicate an unusual nutrition metabolism. Nature 193:616–620.

Stainthorpe, A. C., V. Lees, G. P. C. Salmond, H. Dalton, and J. C. Murrell. 1990. The methane monoxygenase gene cluster of Methylococcus capsulatus (Bath). Gene 91:27–34.

Stainthorpe, A. C., V. Lees, G. P. Salmond, H. Dalton, and J. C. Murrell. 1991. Screening of obligate methanotrophs for soluble methane monooxygenase genes. FEMS Microbiol. Lett. 70:211–216.

Starostina, N. G., and N. I. Pashkova. 1993. Interactions between populations in a 3-component mixed culture of methanotrophic and lytic bacteria. Microbiologiya (English Translation) 62:213–218.

Strauss, D. G., and U. Berger. 1983. Methylosin A and B, pigments from *Methylosinus trichosporium*. J. Basic Microbiol. 23:661–668.

Sullivan, J. P., D. Dickinson, and H. A. Chase. 1998. Methanotrophs, Methylosinus trichosporium OB3b, sMMO, and their application to bioremediation. Crit. Rev. Microbiol. 24:335–373.

Sundh, I., M. Nilsson, and P. Borga. 1997. Variation in microbial community structure in two boreal peatlands as determined by analysis of phospholipid fatty acid profiles. Appl. Environ. Microbiol. 63:1476–1482.

Sutherland, I. W., and A. F. D. Kennedy. 1986. Comparison of bacterial lipopolysaccharides by high performance liquid chromatography. Appl. Environ. Microbiol. 52:948–950.

Sutherland, I. W., and C. L. MacKenzie. 1977. Glucan common to the microcyst walls of cyst-forming bacteria. J. Bacteriol. 129:599–605.

Suzina, N. E., and B. A. Fikhte. 1977. A new type of surface ultrastructure observed in methane-oxidizing microorganisms. Dokl. Akad. Nauk. SSSR 234:470–471.

Takeda, K. 1988. Charcateristics of a nitrogen-fixing methanotroph, Methylocystis T-1. Antonie van Leeuwenhoek 54:521–534.

Takeda, K., S. Motomatsu, Y. Hachiya, S. Fukuoka, and Y. Takahara. 1974. Characterization and culture conditions for a methane-oxidizing bacteria. J. Ferm. Technol. 52:793–798.

Tartakovsky, B., C. B. Miguez, L. Petti, D. Bourque, D. Groleau, and S. R. Guiot. 1998. Tetrachloroethylene dechlorination, using a consortium of co-immobilized methanogenic and methanotrophic bacteria. Enzyme Microb. Technol. 22:255–260.

Tellez, C. M., K. P. Gaus, D. W. Graham, R. G. Arnold, and R. Z. Guzman. 1998. Isolation of copper biochelates from Methylosinus trichosporium OB3b and soluble

methane monooxygenase mutants. Appl. Environ. Microbiol. 64:1115–1122.

Toukdarian, A. E., and M. E. Lidstrom. 1984a. DNA hybridization analysis of the nif region of two methylotrophs and molecular cloning of nif-specific DNA. J. Bacteriol. 157:925–930.

Toukdarian, A. E., and M. E. Lidstrom. 1984b. Nitrogen metabolism in a new obligate methanotroph, "Methylosinus" strain 6. J. Gen. Microbiol. 130:1827–1837.

Trotsenko, Y. A., N. V. Doronina, and P. Hirsch. 1989. Genus Blastobacter. J. T. Staley, M. P. Byrant, N. Pfennig and J. G. Holt, Bergey's Manual of Systematic Bacteriology. Williams and Wilkins Co. Baltimore, 3:1963–1968.

Tschantz, M. F., J. P. Bowman, P. R. Bienkowski, T. L. Donaldson, J. M. Strong-Gunderson, A. V. Palumbo, and G. S. Sayler. 1995. Methanotrophic TCE biodegradtion in a multi-stage bioreactor. Environ. Sci. Technol. 29:2073–2082.

Tsien, H., and R. S. Hanson. 1992. Soluble methane monooxygenase component B gene probe for identification of methanotrophs that rapidly degrade trichloroethylene. Appl. Environ. Microbiol. 58:953–960.

Tsuji, K., H. C. Tsien, R. S. Hanson, S. R. dePalma, R. Scholtz, and S. LaRoche. 1990. 16S ribosomal RNA sequence analysis for determination of phylogenetic relationship amongst methylotrophs. J. Gen. Microbiol. 136:1–10.

Urakami, T., and K. Komagata. 1986a. Cellular fatty acid composition and coenzyme Q system in Gram-negative methanol-utilizing bacteria. J. Gen. Appl. Microbiol. 25:343–360.

Urakami, T., and K. Komagata. 1986b. Emendation of Methylobacullus Yordy and Weaver 1977, a genus for methanol-utilizing bacteria. Int. J. Syst. Bacteriol. 36:502–511.

Vela, G. R., and O. Wyss. 1964. Improved stain for the visualization of Azotobacter encystment. J. Bacteriol. 87:476–477.

Warner, P. J., J. W. Drozd, and I. J. Higgins. 1983. The effect of amino acids and amino acid analogues on the growth of an obligate methanotroph, Methylosinus trichosporium OB3b. J. Chem. Technol. Biotechnol. 33B:2934–2935.

Weaver, T. L., M. A. Patrick, and P. R. Dugan. 1975. Whole-cell and membrane lipids of the methylotrophic bacterium Methylosinus trichosporium. J. Bacteriol 123:602–605.

Wendlandt, K. D., M. Jechorek, J. Helm, and U. Stottmeister. 1998. Production of PHB with a high molecular mass from methane. Pol. Degr. Stab. 59:191–194.

Whittenbury, R., and H. Dalton. 1981. The Methylotrophic Bacteria. P. Starr, H. Stolph, H. G. Truper, A. Blaows and H. G. Schlegel, The Prokaryotes. Springer-Verlagm KG. Berlin, 894–902.

Whittenbury, R., and N. R. Krieg. 1984. Family IV. Methylococcaceae. N. R. Krieg and J. G. Holt, Bergey's Manual of Sytematic Bacteriology. Williams and Wilkins Co. Baltimore, 256–261.

Whittenbury, R., S. L. Davies, and J. F. Davey. 1970a. Exospores and cysts formed by methane-utilizing bacteria. J. Gen. Microbiol. 61:219–226.

Whittenbury, R., K. C. Phillips, and J. F. Wilkinson. 1970b. Enrichment, isolation and some properties of methane-utilizing bacteria. J. Gen. Microbiol. 61:205–218.

Zhivotchenko, A. G., E. S. Nikonova, and M. H. Jorgensen. 1995. Effect of fermentation conditions on N-2 fixation by Methylococcus capsulatus. Biopr. Engineer. 14:9–15.