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Methanotrophic Bacteria

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INTRODUCTION

Methanotrophic bacteria, or methanotrophs, are a subset of a physiological group of bacteria known as methylotrophs. Methanotrophic bacteria are unique in their ability to utilize methane as a sole carbon and energy source. Methylotrophic bacteria are those aerobic bacteria that utilize one-carbon compounds more reduced than formic acid as sources of carbon and energy and assimilate formaldehyde as a major source of cellular carbon (20–22, 112, 152, 155, 232, 415, 416). Methylotrophic bacteria utilize a variety of different one-carbon compounds including methane, methanol, methylated amines, halomethanes, and methylated compounds containing sulfur (20–22, 112, 156, 232). Some cleave methyl groups from organic compounds including choline (111a) or the pesticide carbofuran (382) and utilize them as sole sources of carbon and energy. Bacteria that utilize formate, cyanide, and carbon monoxide have different modes of metabolism including pathways for the assimilation of one-carbon units.

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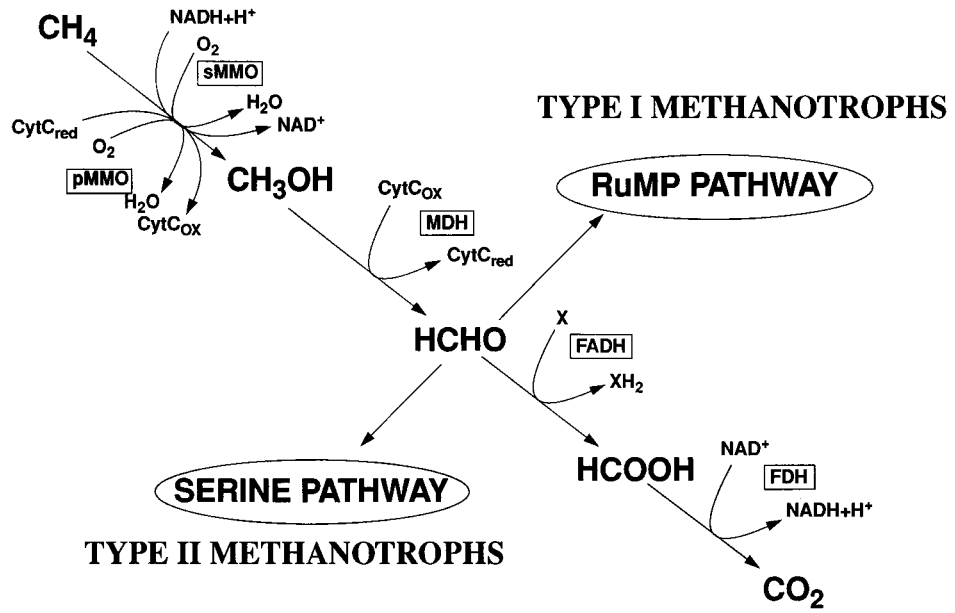

FIG. 1. Pathways for the oxidation of methane and assimilation of formaldehyde. Abbreviations: CytC, cytochrome *c*; FADH, formaldehyde dehydrogenase; FDH, formate dehydrogenase.

The use of enzymes known as methane monooxygenases to catalyze the oxidation of methane to methanol is a defining characteristic of methanotrophs. Figure 1 illustrates the metabolism of substrates by methanotrophs, the common features of their metabolism including the central role of formaldehyde as an intermediate in catabolism and anabolism, and the unique pathways employed for the synthesis of intermediates of central metabolic routes. The two pathways for formaldehyde assimilation found in methanotrophic eubacteria are shown in Fig. 2 and 3. Yeast strains that grow on methanol utilize another pathway known as the dihydroxyacetone pathway for formaldehyde assimilation (22, 223).

Methane is the most stable carbon compound in anaerobic environments and is a very important intermediate in the reactions that eventually lead to the mineralization of organic matter (95). Methane escapes from anaerobic environments to the atmosphere when it is not oxidized by methanotrophs. The release of methane to the atmosphere results in an increased rate of global warming and causes other changes in the chemical composition of the atmosphere (119, 120, 230) that are described later in this review. Söhngen in 1906 (356) recognized that methane was produced in large amounts and suggested that the low atmospheric concentrations of this gas were due to its oxidation by microbes. He isolated the first methane-oxidizing bacterium and named it *Bacillus methanicus*. The oxidation of methane is now known to occur in both aerobic and anaerobic environments, although little has been published about the microbiology or biochemistry of anaerobic methane oxidation. Bacteria that utilize methane, together with some chemolithotrophic bacteria, form the base of a food chain that is independent of photosynthesis near cold gas seeps and hydrothermal vents in the ocean and perhaps undiscovered terrestrial and freshwater environments (64–66, 70, 128, 129, 183, 224).

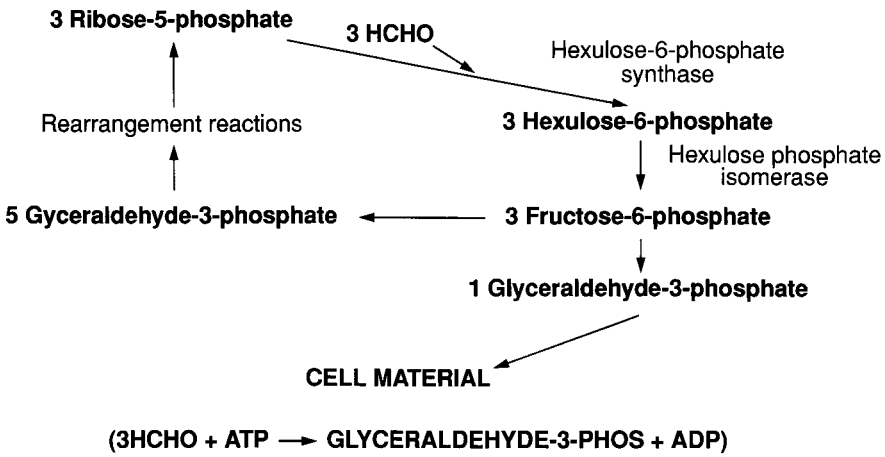

FIG. 2. RuMP pathway for formaldehyde fixation. The reactions catalyzed by the unique enzymes of this pathway, hexulose-6-phosphate synthase and hexulose-phosphate isomerase, are indicated.

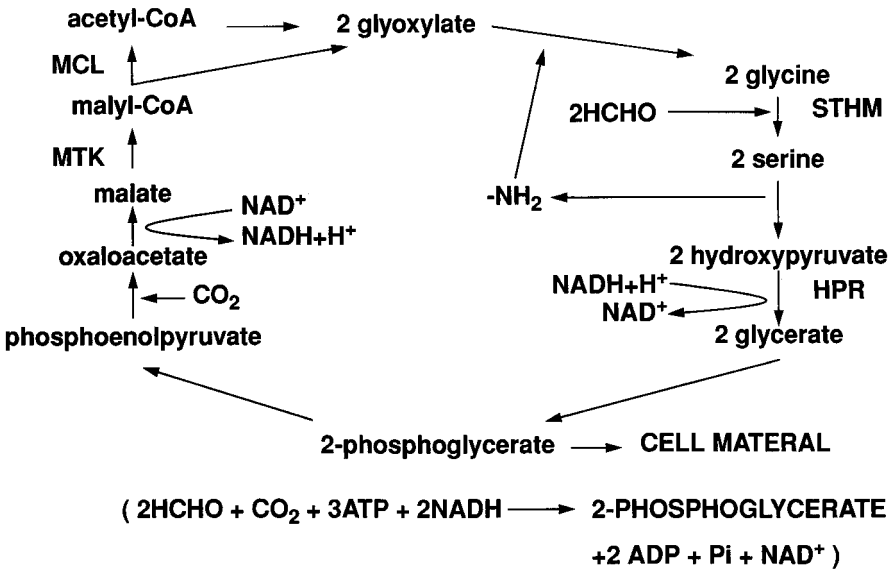

FIG. 3. Serine pathway for formaldehyde fixation. Unique reactions catalyzed by serine hydroxymethyl transferase (STHM), hydroxypyruvate reductase (HPR), malate thiokinase (MTK), and malyl coenzyme A lyase (MCL) are identified.

Methane monooxygenases present in aerobic methanotrophic bacteria exhibit a striking lack of substrate specificity, resulting in the fortuitous metabolism of a very large number of compounds including xenobiotic chemicals (60, 80, 95, 96, 101, 133, 150, 175, 176, 182, 223, 236, 240, 283, 284, 287, 288, 294, 386). Because of the ability of methanotrophs to catalyze a large number of biotransformations, they have attracted the interest of scientists involved in the development of biological methods for degradation of toxic chemicals (bioremediation) and in the use of bacteria containing methane monooxygenases for the production of chemicals with commercial value (154, 176, 193, 223).

This review describes the taxonomy and physiology of methanotrophic bacteria as a prelude to reviewing methods for detection and measurement of the distribution of different methanotrophs in a variety of environments. An overview of global methane cycles, the ecology of methanotrophs, and their interactions with other biota in these cycles is also presented. Finally, the potential role of methanotrophs in remediation of environments contaminated with hazardous chemicals is considered.

PHYSIOLOGY AND TAXONOMY OF METHANOTROPHIC BACTERIA

Taxonomy of Methanotrophic Bacteria

Although the first methanotroph was isolated in 1906 (356), it was not until Whittenbury and his colleagues (417) isolated and characterized over 100 new methane-utilizing bacteria that the basis for the current classification of these bacteria was established. These researchers proposed the separation of methane-utilizing bacteria into five groups (proposed genera) based on morphological differences, types of resting stages formed, the fine structures of intracytoplasmic membranes, and some physiological characteristics (415–417). The genera proposed by Whittenbury et al. (415–417), *Methylomonas*, *Methylobacter*, *Methylococcus*, *Methylocystis*, and *Methylosinus*, are similar to those currently accepted except for the addition of one new genus, *Methylomicrobium* (48, 49). The methanotrophs were separated into two assemblages by the criteria shown in Table 1: type I, including the genera *Methylomonas* and *Methylobacter*, and type II, including the genera *Methylosinus* and *Methylocystis* (141, 155, 415, 416). A new group, type X, was added to accommodate methanotrophs similar to *Methylococcus capsulatus* that, like type I methanotrophs, utilized ribulose monophosphate (RuMP) as the primary pathway for formaldehyde assimilation. Type X methanotrophs were distinguished from type I methanotrophs because they also possessed low levels of enzymes of the serine pathway ribulosebisphosphate carboxylase, an enzyme present in the Calvin-Benson cycle (414–416). They grew at higher temperatures than type I and type II methanotrophs and possessed DNA with a higher moles percent G+C content than that of most type I methanotrophs (141, 147, 155, 415, 416).

Recently, Bowman and his colleagues (48, 49) have compared approximately 124 phenotypic characteristics of 136 methanotrophic bacteria including several new isolates. Numerical taxonomic evaluation, DNA-DNA hybridization, phospholipid fatty acid (PLFA) composition analysis, genomic physiochemical properties, and phylogenetic relationships were used to clarify genus and species interrelationships (47–49). They concluded that the type I methanotrophs included three broadly homologous clusters of species and proposed that the family *Methylococcaceae* should contain the genera *Methylococcus*, *Methylomicrobium*, *Methylobacter*, and *Methylomonas* as redefined in their publications (48, 49). Some features that distinguish genera of type I methanotrophs are given in Table 2. The type II methanotrophs contained closely related groups belonging to the validated species in the genera *Methylocystis* and *Methylosinus* (48, 49). Some features that distinguish genera of type II methanotrophs are provided in Table 3.

Phylogenetic relationships among methylotrophic bacteria have been investigated by 5S rRNA and 16S rRNA sequence analysis (48, 52, 55, 59, 135, 155, 389). The results of these analyses indicated that the methylotrophic bacteria which utilize the serine pathway for formaldehyde assimilation form a distinct assemblage within the alpha-subdivision of the *Proteobacteria* (48, 52, 59, 153, 155, 388) (Fig. 4). The family *Methylococcaceae* formed a distinct branch within the gamma-

TABLE 1. Characteristics of type I, type II, and type X methanotrophs

Characteristic	Type I	Type II	Type X
Cell morphology	Short rods, usually occur singly; some cocci or ellipsoids	Crescent-shaped rods, rods, pear-shaped cells, sometimes occur in rosettes	Cocci, often found as pairs
Growth at 45°C	No	No	Yes
G+C content of DNA (mol%)	49–60	62–67	59–65
Membrane arrangement			
Bundles of vesicular disks	Yes	No	Yes
Paired membranes aligned to periphery of cells	No	Yes	No
Nitrogen fixation	No	Yes	Yes
Resting stages formed			
Exospores	No	Some strains	No
Cysts	Some strains	Some strains	Some strains
RuMP pathway present	Yes	No	Yes
Serine pathway present	No	Yes	Sometimes
Ribulose-1,5-bisphosphate carboxylase present	No	No	Yes
Major PLFAs	14:0, 16:1ω7c, 16:1ω5t	18:1ω8c	16:0, 16:1ω7c
Proteobacterial subdivision	Gamma	Alpha	Gamma
Phylogenetic signature probe(s)[a] *(50)*	1041 (5′-CTCCGCTATCTCTAACAGATT-3′), 1035 (5′-GATTCTCTGGATGTCAAGGG-3′), MM650 (5′-CCTCTACTCAACTCTAGT-3′), MM850 (5′-TACGTTAGCTCCACCACTAA-3′)	1034 (5′-CCATACCGGACATGTCCAAAGC-3′)	No specific probe has been tested

[a] Phylogenetic signature probes 1041 and 1035 will not hydize with 16S rRNAs from members of the genus *Methylomonas*. Probes MM650 and MM850 have been employed to detect some species in this genus (180).

subdivision of the *Proteobacteria*, while the non-methane-utilizing methylotrophs that employ the RuMP pathway for formaldehyde assimilation were found within the beta-subdivision of the *Proteobacteria* (Fig. 4 and 5) (49, 52, 59, 153, 155, 388). Bowman et al. (49) proposed that the new genus *Methylomicrobium* should be added in addition to those proposed by Whittenbury et al. (415–417). This new genus, as proposed, includes some cyst-forming, nonpigmented species which contain distinct phospholipid profiles. These bacteria were previously classified as members of the genera *Methylobacter* and *Methylomonas*. The phylogenetic relationships among members of the *Methylococcaceae* are shown in Fig. 5. The characteristics that distinguish genera of the family *Methylococcaceae* are provided in Table 2.

The 16S rRNA of a methanotrophic endosymbiont found in the gill tissues of a mytilid mussel growing near hydrocarbon cold seeps on the Louisiana Slope in the Gulf of Mexico has been sequenced (114). This uncultured bacterium forms a subbranch within the family *Methylococcaceae* (Fig. 5) and may represent a new genus (49, 114).

Analysis of the 16S rRNA sequences of methanotrophs and methylotrophs that do not utilize methane has permitted the identification of target sequences within the 16S rRNA molecules unique to members of the family *Methylococcaceae* (except for some species of the genus *Methylomonas*) and to the serine pathway (type II) methanotrophs found within the alpha-subdivision of the *Proteobacteria* (55). Oligonucleotide probes that are complementary to these target sequences (Table 1) can be used to detect 16S rRNAs extracted from environmental samples that contain uncultured methanotrophs (46, 55, 153, 180).

Gas chromatography or gas chromatography-mass spectrometry analyses of PLFAs extracted from cultures have provided useful fingerprints for taxonomy and identification of methanotrophs (47, 141, 149, 245, 274, 322, 353). The type I and type X methanotrophs possess 16-carbon fatty acids as the dominant PLFAs, while 18-carbon fatty acids are most abundant in type II methanotrophs (Table 2). Guckert et al. (149) described the application of membrane PLFA analysis as phenotypic markers for methylotrophs by comparing phylogenetic relationships derived from rRNA sequences with relationships arrived at by detailed comparisons of fatty acid chain length and the positions and geometries of double bonds. Members of the genus *Methylomonas* contained mostly 14:0 (19 to 25%) and 16:1ω8c (26 to 41%) fatty acids while *Methylococcus capsulatus* strains contained primarily 16:0 (33 to 56%) and 16:1ω7c (4 to 12%) fatty acids. Type II methanotrophs contained high levels of 18:1ω8c (53 to 74%) and 18:1ω7c (15 to 38%) fatty acids. Because of the unusual nature of the last two fatty acids, they serve as useful environmental markers for type II methanotrophs (47, 48, 153, 274). The strain relationships derived from comparisons of PLFA profiles compared favorably with those derived from 5S and 16S rRNA sequence comparisons (149, 274). These phospholipid profiles are stable regardless of the intracytoplasmic membrane content and the carbon and energy source for facultative methanotrophs (149).

Nearly all methane-utilizing bacteria are obligate methanotrophs (20, 155, 415–417). There have been a few descriptions of facultative methanotrophs and restricted facultative methanotrophs that can be trained to grow on multicarbon compounds (152, 209, 294, 435, 436). *Methylobacterium organophilum* XX was reported to grow with methane as well as a variety of multicarbon compounds as sole sources of carbon and energy (297–299). Recent studies have shown that the restriction fragments produced by digestion of DNAs from methane-grown cells and cells grown in nutrient broth were

TABLE 2. Characteristics that distinguish genera of type I methanotrophs (the family *Methylococcaceae*)[a]

Characteristic	*Methylomonas*	*Methylobacter*	*Methylococcus*	*Methylomicrobium*
Cell morphology	Rods	Cocci, ellipsoidal, or fat rods	Cocci or ellipsoidal	Rods
DNA base composition (mol% G+C)	51–59	49–60	59–66	NA[a]
Genome mol wt	$(1.8–2.3) \times 10^9$	ND[b]	ND	ND
Dominant PLFA	14:0, 16:1ω8c	16:1ω7c, 16:1ω5t	16:0, 16:1ω7c	16:1ω5t, 16:1ω7c
Carotenoids produced	+	−	−	−
Motility	+	Variable	Variable	Variable
Cyst formation	+	+	+	−
Cyst desiccation				
Sensitive	+	−	+	−
Resistant	−	+	−	−
Ribulose-1,5-diphosphate carboxylase	−	−	+	−
Nitrogen fixation	−	−	+	−
Growth at 45°C	−	−	+	−
Brown or yellow pigmentation	−	+	+	−
Species	*M. aurantiaca*, *M. fodinarum*, *M. methanica*	*M. albus*, *M. marinus*, *M. whittenburyi*	*M. capsulatus*, *M. thermophilus*	*M. agile*, *M. album*, *M. pelagicum*

[a] Data from references 48 and 49. All members of this group are gram-negative, strictly aerobic, obligate methylotrophs that employ the RuMP pathway for formaldehyde assimilation. They possess intracytoplasmic membranes which appear as stacked vesicular disks. If motile, they possess a single polar flagellum. Catalse is produced, and most have an incomplete tricarboxylic acid cycle and lack α-ketoglutarate dehydrogenase. All are members of the gamma-subdivision of the *Proteobacteria.*

[b] ND, not determined.

indistinguishable, as were the sizes of the restriction fragments that hybridized with the *mxaF* gene, which encodes the large subunit of methanol dehydrogenase (396a). Moreover, PLFA analysis indicated that the bacteria which grew on methane and methanol were indistinguishable (149, 274). *M. organophilum* XX is found to cluster with other pink-pigmented facultative methylotrophs within the alpha-subdivision of the *Proteobacteria* by phylogenetic analysis (55, 389) (Fig. 4). *Methylomonas* sp. strain 761 is an unusual type I methanotroph, which possesses a complete tricarboxylic acid cycle and can be adapted to grow on glucose as the sole source of carbon and energy (434–436). During growth on glucose in the absence of methane for more than 10 generations, it maintained the ability to oxidize methane at the same specific rates found in methane-grown cells (435, 436). The results of phylogenetic analyses (Fig. 4 and 5) indicated that this bacterium is a distinct species most closely related to the orange-pigmented species of the genus *Methylomonas* (48, 55, 389).

Acceptance of the existence of facultative methanotrophs by scientists who study methanotrophic bacteria is not universal. However, the question of their existence should be considered in light of the properties of soil bacteria which oxidize atmospheric methane that are discussed later in this review.

Physiology of Methanotrophic Bacteria

Understanding the factors that control methane metabolism and the ecology of methanotrophic bacteria requires knowledge of the physiology of different groups of methanotrophs. The differences in the enzyme systems employed by different genera and species for the oxidation of methane, the pathways used for the assimilation of one-carbon units into central metabolic routes, the chemical composition of cellular constituents, regulatory mechanisms that control the metabolism of one-carbon compounds, and the nutritional responses of different methanotrophs will determine the abilities of different bacteria to compete in different habitats. Therefore, it is appropriate to review the current knowledge of the physiology of different groups of methanotrophs insofar as it relates to their ability to survive, grow, and oxidize methane in different environments and their capacity to degrade environmental pollutants that are considered hazardous to human or ecosystem health.

Methane oxidation. The oxidation of methane by aerobic methanotrophs is initiated by methane monooxygenases (MMOs) (20, 21, 98–100, 239), which are classical monooxygenases that utilize two reducing equivalents to split the O—O bonds of dioxygen (98, 239). One of the oxygen atoms is reduced to form H_2O, and the other is incorporated into methane to form CH_3OH. Two forms of MMOs have been found in methanotrophic bacteria (21, 98, 99, 307). One form, a soluble MMO (sMMO), utilizes NADH + H^+ as an electron donor and remains soluble after centrifugation of cell extracts at 150,000 × *g* for 75 min (99, 239). The sMMO has been purified from a number of type II and X methanotrophs, including *Methylo-*

TABLE 3. Characteristics that distinguish genera of type II methanotrophs[a]

Characteristic	*Methylosinus*	*Methylocystis*
Cell morphology	Vibriod or pyriform	Cocci, curved rods, ellipsoidal
Cyst formation (desiccation sensitive)	−	+
Exospore (bud) formation	+	−
Lysed by 2% (wt/vol) SDS	+	−
Representative species	*M. trichosporium*, *M. sporium*	*M. echinoides*, *M. parvus*, *M. pyriformis*, *M. minimus*

[a] Data from references 48 and 49. All species are gram-negative, strictly aerobic, obligate methylotrophs that use the serine pathway for formaldehyde assimilation. They possess intracytoplasmic membranes which are aligned parallel to the cell wall. They do not grow at 45°C. They contain a complete tricarboxylic acid cycle, lack enzymes of the Calvin-Benson cycle, and fix nitrogen via an aerotolerant nitrogenase system. The major PLFA is 18:1ω8c, and the DNA base compositions range from 62 to 67 mol% G+C.

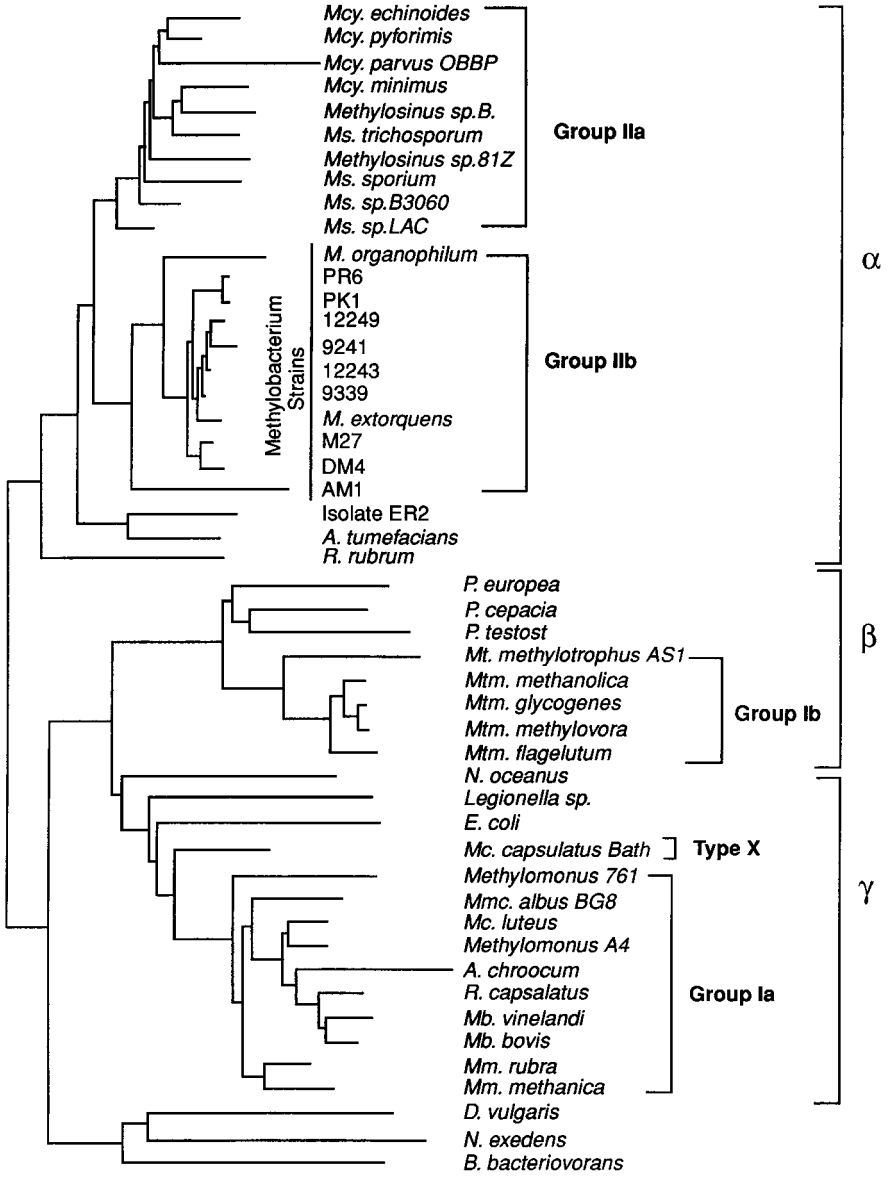

FIG. 4. Unrooted phylogenetic tree showing the relationships among methanotrophic bacteria, other methylotrophic bacteria, and nonmethylotrophic bacteria belonging to the alpha-, beta-, and gamma-subdivisions of the *Proteobacteria*. The abbreviations for genus names of methylotrophic bacteria used in this figure are as follows: *M.*, *Methylobacterium*; *Mcy.*, *Methyloystis*; *Ms.*, *Methylosinus*; *Mb.*, *Methylobacterium*; *Mc.*, *Methylococcus*; *Mp.*, *Methylophilus*; *Mmc.*, *Methylomicrobium*; *Mm.*, *Methylomonas*; *Mtm.*, *Methanomonas*. Group Ia includes the methanotrophic bacteria that employ the RuMP pathway for formaldehyde assimilation, while group Ib includes those methylotrophs that employ the RuMP pathway for formaldehyde fixation but do not oxidize or grow with methane as a source of carbon and energy. Group IIa includes methanotrophic bacteria that employ the serine pathway for formaldehyde assimilation, and group IIb includes those methanotrophic bacteria that utilize the serine pathway for formaldehyde fixation but do not utilize methane. Isolate ER2 is a facultative methylotroph that utilizes methylamine and can degrade the herbicide carbofuran. It does not grow with methane or methanol as the sole source of carbon and energy.

coccus capsulatus (Bath) (type X), *Methylosinus sporium* (type II), *Methylocystis* sp. strain M (type II), and *Methylobacterium* sp. strain CRL26, a bacterium of uncertain taxonomic status (78–80, 98, 100, 102, 134, 146, 175, 228, 239, 269, 296, 304, 305, 368, 427). The sMMOs of these bacteria are very similar (98, 239, 266). They do not contain heme cofactors or any other cofactors encountered previously in oxygenase chemistry (239), and all sMMOs are believed to contain three components. The hydroxylase component is 245 kDa in size, contains nonheme iron, and is an oligomer of three different subunits (α, β, and γ). The B component (15.8 kDa) is a colorless protein with no cofactors, and the reductase component is 38.4 kDa in size and contains flavin adenine dinucleotide and an [Fe_2S_2] cluster. The structure of this enzyme, its reaction kinetics, and the roles of each component in the catalytic cycle have been reviewed elsewhere (102, 239).

The genes encoding the subunits have been cloned and sequenced from two bacteria: *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath) (61, 62, 266, 267, 360–362). The sequences are highly conserved among methanotrophs, as shown by DNA-DNA hybridization reactions between cloned genes encoding the hydroxylase subunits of sMMO and DNA isolated from different species (180, 254, 266, 267, 387). These genes are useful as probes to detect the

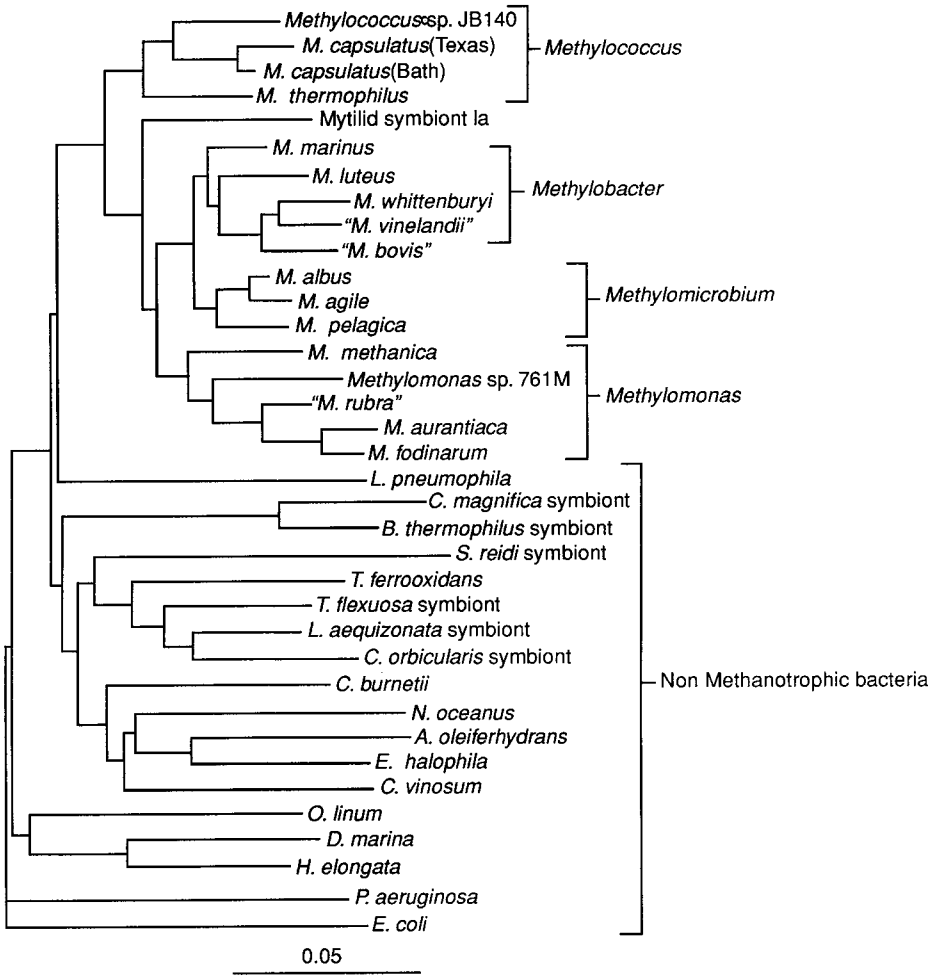

FIG. 5. Unrooted phylogenetic tree showing the relationships among members of the family *Methylococcaceae* and other members of the gamma-subdivision of the *Proteobacteria*. Reproduced and modified from reference 49 with permission.

presence of methanotrophs that contain genes encoding sMMO and to determine if these genes are expressed in different environments.

All known methanotrophs are capable of forming a particulate or membrane-bound MMO (pMMO) that sediments in 75 min at 150,000 × *g* (48, 67, 97–99, 266, 267, 295, 307, 355, 363). Extensive intracytoplasmic membranes and pMMO activity are present only when copper concentrations exceed 0.85 to 1 μmol/g (dry weight) of cells (51, 81, 142, 272, 307, 363, 377, 378). Cells of *Methylomonas margaritae* grown with low levels of copper were devoid of intracytoplasmic membranes, and vesicles were observed only at the periphery of the cells (377, 378) rather than throughout the cytoplasm as observed in cells grown with higher levels of copper. Most of the copper in cells is associated with the membrane fraction when the copper concentration in the medium is below 20 μM (272). Mutants of *Methylosinus trichosporium* that are unable to synthesize pMMO and that synthesize sMMO at concentrations of copper up to 12 μM do not synthesize intracellular membranes (302). It is possible that these mutants are altered in their ability to transport copper.

The pMMO of all methanotrophs studied is easily inactivated in extracts and has not been purified to homogeneity or characterized to the extent that the sMMOs of different methanotrophs have been (67, 355). The pMMO of *Methylococcus capsulatus* (Bath) requires copper for activity (272), and the copper is believed to be present in the active site of the enzyme (67, 351). Three polypeptides with molecular masses of 45, 35, and 26 kDa that are present in the membranes of cells which express pMMO have been associated with this activity (67, 350, 363).

All methanotrophs are believed to be capable of expressing pMMO when grown in the presence of copper (99), but the ability to form sMMO and positive sMMO gene probe hybridizations with DNAs extracted from cells has been observed only in some type II methanotrophs (99, 266–269, 305, 387), the type X methanotroph *Methylococcus capsulatus* (78–80, 98–101, 146), and one type I methanotroph (218). In bacteria that have DNA with sequences complementary to sMMO genes and that express sMMO activity as evidenced by specific assays, sMMO activity is present in cells grown in the presence of less than 0.89 μmol of copper per g (dry weight) of cells (56, 99, 154, 361, 386, 387). The addition of more copper leads to the synthesis of additional intracytoplasmic membranes (81), appearance of the membrane proteins associated with pMMO (99), increased growth yields, and a loss of sMMO activity (226–228).

The sMMO has a broader substrate specificity than the pMMO and other oxygenases do (60, 150, 175). It can be detected in cells of some methanotrophs grown in media with

low levels of copper by the addition of naphthalene to the cell suspensions. Cells containing sMMO rapidly oxidize naphthalene to 1- and 2-naphthols, which can be detected by the addition of tetrazoitized *o*-dianisidine, resulting in the formation of purple diazo dyes with large molar absorbtivities (56). pMMO does not oxidize naphthalene to naphthols and so is unreactive in the assay. This reaction can be used to detect sMMO activity in cell suspensions, in colonies grown on agar plates with low levels of copper, and in methanotrophs recovered from environmental samples. Antigens that cross-react with sMMO antibodies, DNA complementary to the *mmoB* gene, and sMMO activity as determined by the naphthalene oxidation assay have been detected in the following methanotrophs: *Methylococcus capsulatus* (Bath), a type X methanotroph; the type II methanotrophs *Methylosinus sporium*, *Methylosinus trichosporium* OB3b, and *Methylosinus* sp. strain B; some natural isolates identified as type II methanotrophs (387); and one type I methanotroph, *Methylomonas methanica* 68-1 (218). The type II methanotrophs *Methylocystis pyriformis* and *Methylocystis parvis*, as well as type I methanotrophs with the one exception noted above, lack sMMO activity and sMMO-cross-reacting antigens and do not hybridize to a *mmoB* gene probe (218, 387).

It has been observed that most type I methanotrophs require higher levels of copper for growth than do type II methanotrophs and *Methylococcus capsulatus* (type X), presumably because they require higher levels of copper for pMMO activity and lack genetic information for sMMO synthesis (145, 267).

It is difficult at present to draw firm conclusions about the taxonomic and phylogenetic relationships among strains of methanotrophs that are capable of synthesizing sMMO when grown with limiting copper concentrations. It is not yet clear if the ability to synthesize sMMO is confined to a certain taxonomic or phylogenetic groups or if all members of some taxonomic and phylogenetic groups synthesize this enzyme. It is a characteristic that appears to be most common among members of the genera *Methylosinus* and *Methylococcus* but is not strictly confined to bacteria classified as members of these genera.

Cells of methanotrophs that contain pMMO have higher growth yields on methane and have greater affinity for methane than do cells that contain sMMO, because the sMMO-catalyzed reaction requires $NADH + H^+$ as an electron donor (225–228, 239) while the pMMO employs a higher-potential electron donor. The supply of reduced NAD^+ is often growth limiting when methane is used as a substrate (225–228, 307). The synthesis of sMMO by some methanotrophs may be a survival mechanism in the many environments where copper limits the growth of methanotrophs capable of synthesizing only pMMO.

Methanol oxidation. Methanol from endogenous (methane oxidation via MMO) and exogenous sources (pectin and lignin degradation are examples) is oxidized to formaldehyde by a periplasmic methanol dehydrogenase (MDH) in gram-negative methylotrophs (21, 24, 105, 136, 423). MDH is an $\alpha_2\beta_2$ tetramer of large (60- to 67-kDa) and small (8.5-kDa) subunits (24). It is a quinoprotein; each tetramer contains 2 mol of pyrroloquinoline quinone and 1 mol of calcium (24, 105, 136). Electrons are transferred from MDH to cytochrome c_L, an atypical cytochrome which serves as the specific electron acceptor for MDH (21, 24, 136, 310). Cytochrome c_L is then oxidized by a typical class I cytochrome *c* (cytochrome c_H), which is also specific for the oxidation of methanol (21, 24, 112). The MDH and the two cytochromes are soluble and are located in the periplasm of gram-negative methylotrophs (23, 136). These proteins are found in large amounts, and mutants lacking any of them fail to grow on methanol, but facultative methylotrophs can be rescued by growth on heterotrophic substrates or methylamines (5, 6, 21, 30, 32, 111a, 150, 161, 266, 267, 278–280). The genes for the large and small subunits of MDH and cytochrome c_L are closely linked on the genomes of the gram-negative methanol utilizers *Paracoccus denitrificans*, *Methylobacterium extorquens*, and *Methylobacterium organophilum* XX, as well as the methanotrophs *Methylosporovibrio methanica* 81Z and *Methylomonas* sp. strain A4 (30, 32, 157, 163, 233, 234, 243, 266, 267, 278, 399). The genes encoding the large subunit of MDH (*mxaF*) have been cloned from a variety of methylotrophs (18, 19, 32, 161–163, 233, 236, 242–244, 266, 267, 278, 367). The *mxaF* sequences from the facultative methylotrophs *Methylobacterium organophilum* XX, *Methylobacterium extorquens* AM1, and *Paracoccus denitrificans* are highly conserved (30, 157, 161–163, 233, 242–244, 266, 267, 278). Partial sequences are available for other methylotrophic bacteria (236). Genes cloned from one methylotroph often complement defective genes in another, antibodies produced against an enzyme from one methylotroph cross-react with MDHs from other methylotrophs, and genes cloned from facultative methylotrophs hybridize with DNA from a variety of type I and type II methanotrophs as well as with DNA from methylotrophs that do not use methane (32, 111a, 157, 254).

Methanol is oxidized via an NAD-linked methanol dehydrogenase in gram-positive methylotrophs and via a methanol oxidase system in methanol-oxidizing yeast species, but these enzymes have not been detected in gram-negative methanotrophic bacteria (112).

The regulation of MDH synthesis involves very complex regulatory circuits in facultative methylotrophs and at least one methanotroph. The literature describing the regulation of MDH synthesis has been extensively reviewed elsewhere (30, 161, 236, 267).

Oxidation of formaldehyde and formate. Most of the reducing power required for the metabolism of methane is produced by the oxidation of formaldehyde via formate to carbon dioxide. There are multiple enzyme systems for the oxidation of formaldehyde to formate in methylotrophs (22, 112). These include NAD(P)-linked aldehyde dehydrogenases that may or may not require reduced glutathione or other cofactors and dye-linked dehydrogenases that are measured by the reduction of dyes such as 2,6-dichlorophenol (112). Formate is oxidized to carbon dioxide by an NAD-dependent formate dehydrogenase in most if not all methanotrophs (22, 112). A cyclic pathway for the oxidation of formaldehyde to carbon dioxide has been described and found to exist in methylotrophs that possess the RuMP pathway for formaldehyde assimilation (22, 308, 309, 372, 431). In this pathway, formaldehyde and ribulose-5-phosphate react to form hexulose-6-phosphate, which is isomerized to fructose-6-phosphate, and this substrate is converted to glucose-6-phosphate, which is in turn oxidized to 6-phosphogluconate. The 6-phosphogluconate is then oxidized to produce carbon dioxide and ribulose-6-phosphate to complete the cyclic pathway for formaldehyde oxidation. The electron acceptors for the two oxidations in the cycle are NAD^+ or $NADP^+$. Most obligate methanotrophs employ the linear pathway for formaldehyde oxidation, while many non-methane-utilizing methylotrophs employ the cyclic pathway as the major route (22, 112, 431).

Assimilation of formaldehyde. Formaldehyde produced from the oxidation of methane and methanol by methanotrophic bacteria is assimilated to form intermediates of the central metabolic routes that are subsequently used for biosynthesis of cell material (22, 112, 308, 309). The two known pathways used by methanotrophic bacteria for the synthesis of multicarbon compounds from formaldehyde are the serine

pathway, in which 2 mol of formaldehyde and 1 mol of carbon dioxide are utilized to form a three-carbon intermediate, and the RuMP cycle for the assimilation of 3 mol of formaldehyde to form a three-carbon intermediate of central metabolism (20, 22, 111a, 223, 308, 309). In the latter pathway, all cellular carbon is assimilated at the oxidation level of formaldehyde.

The RuMP pathway was first described by Quayle and his colleagues (197, 204, 372). In this pathway (Fig. 2), formaldehyde is combined with ribulose monophosphate via an aldol condensation to form hexulose-6-phosphate in a reaction catalyzed by hexulosephosphate synthase (HPS). Hexulose-6-phosphate is converted to fructose-6-phosphate by hexulosephosphate isomerase (HPI). These two enzymes are unique to the metabolism of one-carbon compounds by type I and type X methanotrophs and some other methylotrophs that do not utilize methane.

HPS from the obligate type X methanotroph *Methylococcus capsulatus* (Bath) is a very large (310-kDa), hexameric, membrane-bound enzyme, whereas enzymes from bacteria that do not utilize methane appear to be soluble, smaller (15.5- to 45-kDa), dimeric or monomeric enzymes (26, 112, 200). Hexulosephosphate isomerase is soluble in methanotrophs (26, 127, 200). Although mutants lacking HPS and HPI have been isolated, the genes encoding the proteins have not been cloned or sequenced, and it is not known if the amino acid sequences of these enzymes or the nucleotide sequences of the genes that encode them are conserved in methylotrophic bacteria (266, 267).

In the second (cleavage) part of the RuMP pathway, the fructose-6-phosphate produced in the first part of the pathway is converted to 2-keto-3-deoxy-6-phosphogluconate and this compound is cleaved to yield pyruvate and glyceraldehyde-3-phosphate. In the third (rearrangement) part of the pathway, the glyceraldehyde phosphate and fructose-6-phosphate molecules undergo a series of reactions which lead to the regeneration of ribulose-5-phosphate to complete the pathway (22, 112, 308). The enzymes of the cleavage pathway are found in heterotrophic bacteria that employ the Entner-Doudoroff pathway for glucose metabolism, and the rearrangement reactions are identical to those found in some autotrophic bacteria. It has been suggested that the RuMP pathway was an evolutionary precursor of the Calvin cycle (309, 414).

Gram-negative methylotrophic bacteria that utilize the serine pathway as the primary route for formaldehyde fixation are related to other gram-negative bacteria in the alpha-subdivision of the *Proteobacteria* (52, 55, 389). The type II methanotrophic bacteria form a distinct cluster within this subdivision (Fig. 4) (22, 112).

In the first reaction of the serine pathway, formaldehyde reacts with glycine to form serine. This reaction is catalyzed by serine hydroxymethyltransferase. Two such enzymes, one for assimilation of formaldehyde and one for biosynthesis of glycine from serine, are known in *Methylobacterium extorquens* AM1 and *Methylobacterium organophilum* XX (279, 308). Serine then undergoes transamination with glyoxylate as the amino group acceptor to produce hydroxypyruvate and glycine. Hydroxypyruvate is reduced to glycerate by hydroxypyruvate reductase. Glycerate kinase catalyzes the addition of a phosphate group from ATP to produce 2-phosphoglycerate. The conversion of 2-phosphoglycerate to phosphoenolpyruvate, the fixation of carbon dioxide catalyzed by phosphoenolpyruvate carboxylase, and the reduction of oxaloacetate to malate follow reactions common to many heterotrophic bacteria. Malyl coenzyme A is formed in a reaction catalyzed by malate thiokinase and is cleaved by malyl coenzyme A lyase. These two enzymes, as well as hydroxypyruvate reductase, and glycerate-2-kinase, are uniquely present in methylotrophs that contain the serine pathway (22, 30, 112, 232, 266, 308).

In the second part of the pathway, acetyl coenzyme A is converted to glyoxylate by the glyoxylate cycle or another unknown pathway that occurs primarily in facultative methylotrophs that do not contain isocitrate lyase, a key enzyme of the glyoxylate cycle (22, 111a, 112).

Genes encoding serine hydroxymethyltransferase (*glyA*), hydroxypyruvate reductase (*hprA*), phosphoenolpyruvate carboxylase (*ppcA*), malyl coenzyme A lyase (*mclA*), malate thiokinase (*mtkA* and *mtkB*), glycerate kinase, serine glyoxylate aminotransferase (*sgaA*), and an unknown product required for the conversion of acetyl coenzyme A to glyoxylate have been identified in clones of *Escherichia coli* carrying vectors with inserts of DNA from the pink-pigmented facultative methylotroph *Methylobacterium extorquens* AM1 (27, 71–74, 262). The gene for serine hydroxymethyltransferase was cloned from the obligate methylotroph *Hyphomicrobium methylovorum* GM2. Although the genes for phosphoenolpyruvate carboxylase, hydroxypyruvate reductase, serine glyoxylate aminotransferase, malate thiokinase, and malyl coenzyme A lyase have been shown to be linked to each other and to some other genes required for the expression of methanol dehydrogenase on the genome of *Methylobacterium extorquens* AM1 (71–74), it is not known if the linkage relationships or sequences of genes encoding unique enzymes of this assimilation pathway are conserved in methylotrophs (236). If the genes are conserved, they could serve as valuable gene probes for methylotrophs which utilize the serine pathway for formaldehyde assimilation.

It can be concluded that unique enzymes are found in the pathways for methane oxidation and the assimilation of C_1 units in methanotrophs. These include sMMO and pMMO, which are unique to methanotrophs, and MDH subunits and cytochromes required for methanol metabolism. Five structural genes encode proteins found in sMMO, and two unique genes are required to encode MDH subunits. Several of these genes, including the MMO hydroxylase subunit genes and the genes encoding the large subunit of MDH, appear to be highly conserved (30, 157, 254, 266). It seems quite likely that genes for serine hydroxymethyltransferase, malate thiokinase from the serine pathway, and hexulose phosphate synthase and isomerase from the RuMP cycle could also be unique to methanotrophs, although the extent of conservation of these genes is not known. Genes from one methylotroph, including uncultured organisms in environmental samples, can be recognized by probing with a heterologous gene probe or an oligonucleotide complementary to a conserved region of the genes (13, 254, 267, 387). Sequences of genes recovered from environmental samples can then be used as aids in constructing taxonomic relationships among uncultured and well-characterized methylotrophs.

Physiological Similarities between Methanotrophic and Ammonia-Oxidizing Bacteria

Autotrophic ammonia-oxidizing bacteria are a phylogenetically diverse group of microorganisms. *Nitrosococcus mobilis* and species *Nitrosovibrio*, *Nitrosomonas*, *Nitrosospira*, and *Nitrosolobus* form a coherent phylogenetic group interpreted as a single family in the beta-subdivision of the *Proteobacteria*, while *Nitrosococcus* strains are found in the gamma-subdivision (168). As mentioned previously, methanotrophs are equally diverse in their phylogenetic relationships, forming two clusters in the alpha- and gamma-subdivisions (48, 52, 55, 389). However, these bacteria are very similar in the ways they oxi-

dize methane and ammonia. These similarities are important when describing microfloras that oxidize methane and ammonia in the same environments.

Ammonia-oxidizing bacteria are chemoautotrophs that oxidize ammonia to nitrite to obtain energy for carbon dioxide fixation via the Calvin-Benson cycle (405), whereas formaldehyde is the major source of carbon for synthesis of cell material by methanotrophs. Both groups of bacteria fortuitously oxidize a variety of compounds. Ammonia and methane oxidizers oxidize methane, ammonia, carbon monoxide, methanol, hydroxylamine, ethylene, propylene, phenol, trichloroethylene, and a few other compounds (25, 33, 96, 150, 176, 185, 186, 283, 284, 288, 386). Both oxidize ammonia and methane as well as several other substrates, although the apparent K_s values for ammonia and methane differ considerably (33, 210, 213). Most of these reactions are known or believed to be catalyzed by MMO and ammonia monooxygenase (AMO) (25, 33, 56, 96, 283, 284, 288). Furthermore, AMO, which initiates the oxidation of ammonia in the ammonia-oxidizing bacteria, and pMMO share several properties. Both use a reductant other than NADH + H^+ (probably a cytochrome *c*) (357, 385, 430), both are highly unstable in cell extracts, both are inhibited by acetylene, and [^{14}C]acetylene covalently labels a membrane polypeptide of 27 kDa in cells of both groups of bacteria (350, 351). The activity of both enzymes in vitro is stabilized by copper, and copper is proposed to be part of the active site of both (122, 272). The AMO of *Nitrosomonas europeae* has been purified, and the sequences of genes believed to encode subunits of AMO of are known (38, 256). The molecular weights and amino acid sequences of the pMMO subunits were inferred from the sequences of cloned genes, because this enzyme has not been purified to homogeneity. Both enzymes appear to contain subunits of 27 and 45 kDa, and the genes encoding the two enzymes share considerable sequence homology (350, 351). The 45-kDa membrane protein that is believed to be associated with the pMMO was purified from three methanotrophic bacteria, *Methylococcus capsulatus* (Bath), *Methylomicrobium albus* BG8, and *Methylobacter marinus* A45, and was shown to be identical in 17 of 20 N-terminal positions. Of the 20 N-terminal amino acids, 14 were identical to those in the N terminus of the AmoB protein of AMO from *Nitrosomonas europaea.*

Clones carrying the gene encoding the 45-kDa peptide of the pMMO of *Methylococcus capsulatus* (Bath) (*pmoB*) were identified with an oligonucleotide probe designed from the N-terminal amino acid sequence of the peptide (350, 351). Other clones believed to carry *M. capsulatus* DNA encoding pMMO subunits were identified with the cloned *pmoB* gene and an internal fragment of the *amoA* gene from *N. europaea*, which encoded a 27-kDa peptide of AMO. These genes were sequenced and were found to be linked on the chromosome. The predicted amino acid sequences of the *pmoA* and *pmoB* genes which encoded 27- and 45-kDa polypeptides were very similar to the gene products of the *amoA* and *amoB* genes (350, 351). DNA-DNA hybridization studies with cloned genes as probes revealed that the *pmo* genes are present in duplicate copies in a variety of methanotrophs and that the *amo* genes are also duplicated in *N. europaea*. The two sets of genes may be differentially expressed at different copper concentrations, and they may encode pMMOs and AMOs with different spectral characteristics (350, 351).

Ammonia-oxidizing bacteria oxidize methane and incorporate carbon dioxide into cell material with ammonia or methane as substrates (185, 401). On a cell dry weight basis, the specific rate of methane oxidation by ammonia oxidizers is less than 5% of the specific rates observed with resting cells of methanotrophs and the K_m for methane of nitrifiers is much higher than the K_m values for methane estimated for methanotrophs (33). Methane inhibits the oxidation of ammonia to nitrite by nitrifying bacteria (33, 37b).

All methane-oxidizing bacteria examined, including endosymbionts of the pogonophore, *Siboglinum poseidoni*, oxidized ammonia to nitrite (33, 48, 96, 258, 334), although the specific rates of ammonia oxidation by methanotrophs were 2 orders of magnitude lower than those of the chemoautotrophic nitrifiers (33). Ammonia is oxidized by both pMMO and sMMO (80, 96, 430), and ammonia inhibits methane oxidation, reducing the growth rate of all methanotrophs tested (417). Megraw and Knowles (257–259) described methane-dependent nitrification in a humisol and postulated that methanotrophs may play significant roles in nitrification in acidic soils that are not favorable for the growth of nitrifiers. Slurries of sediment from Hamilton Harbour, Ontario, Canada, supplemented with 1 to 24 mM methane showed enhanced rates of nitrification compared with unsupplemented slurries (326). At concentrations of methane higher than 84 μM, nitrification was suppressed, perhaps by competition between methanotrophs and ammonia-oxidizing bacteria for oxygen (326).

The oxidation of ammonia and hydroxylamine by methanotrophs is accompanied by the production of small amounts of nitrous oxide (217, 381, 429, 430). Nitrous oxide and methane are both radiatively active or "greenhouse" gases. Ammonia-oxidizing bacteria also produce N_2O during the oxidation of ammonia to nitrite (217, 430).

Methanotrophic bacteria contain hydroxylamine cytochrome *c* oxidoreductase (HAO) activity (23, 49), while methylotrophs that do not oxidize methane do not oxidize hydroxylamine to nitrite (380a). MDH and HAO, present in extracts of *Methylococcus thermophilus*, were separated by ion-exchange chromatography (357). MDH does not oxidize hydroxylamine, demonstrating that MDH is not responsible for hydroxylamine oxidation in this bacterium. The HAO purified from *Methylococcus capsulatus* (Bath) was similar to cytochrome P-460 (430), as is the heme-containing enzyme from *Nitrosomonas europaea*. The HAO of *M. capsulatus* (Bath) also resembled the enzyme of *N. europaea* in its N-terminal amino acid sequence, molecular mass, other physical and kinetic parameters, and stimulation by phenazine methylsulfate (430). HAO may be present in methanotrophs to prevent the accumulation of hydroxylamine fortuitously produced from ammonia by MMO. Hydroxylamine is extremely toxic and mutagenic to bacteria.

The abilities of methanotrophs to oxidize ammonia and of ammonia oxidizers to oxidize methane have led to the hypothesis that methanotrophs may play a role in the oxidation of ammonia in some environments and that ammonia oxidizers may also oxidize methane under the appropriate environmental conditions (96, 326). Estimates of the contributions of each may be possible because 2-pyridine carboxylic acid (picolinic acid) inhibits soil methane oxidation at 500 μM but fails to inhibit the production of nitrate from ammonia in soils at 2 mM (258). The inhibition of methane oxidation by ammonia has profound effects on the ecology of methanotrophs in agricultural soils, forests, and rice paddies as described below.

ROLES OF METHANOTROPHS IN GLOBAL CARBON CYCLES

Atmospheric Methane and Its Role in Global Warming

Methane is the most abundant organic gas in the atmosphere (75, 88, 91, 230, 366, 373). The molar ratio of atmospheric carbon dioxide and methane is approximately 27 at present, and this ratio will decrease to 7.5 in 100 years accord-

ing to a recent assessment (230). Although the current concentration of methane is much lower than the concentration of carbon dioxide in the atmosphere, methane absorbs terrestrial radiation in the 4- to 100-nm region (infrared irradiation) more effectively than does carbon dioxide, and reemission of the absorbed radiant energy causes global warming (230). Therefore, methane is estimated to contribute about 26 times that of carbon dioxide (mole for mole) to climate change (230). During the past century, methane has accounted for 15 to 25% of the thermal trapping while carbon dioxide has contributed 60% (179, 311, 321, 408). Reductions in methane emissions would be 20 to 60 times more effective in reducing the potential warming of the Earth's atmosphere over the next century than would equivalent molar reductions in CO_2 emissions (179, 391).

Since the last major glaciation (about 18,000 years ago), the concentration of methane in the atmosphere has increased from 0.35 to 1.7 ppm. In the last 300 years, the concentration of atmospheric methane increased from 0.75 to 1.7 ppm and has continued to increase by 0.8 to 1.0% per year until recently, when slight decreases in the rate of increase of atmospheric methane have been reported (39, 88, 206, 325, 364–366). It is estimated that the concentration of atmospheric methane will reach 2.1 to 4.0 ppm by the year 2050 (311). Steele et al. (365) have reported that there has been a substantial slowing of the global accumulation rate in northern latitudes (30 to 90°N) between 1983 and 1990.

It has been predicted that increases in methane production in the atmosphere will decrease OH radical concentrations and thus increase the lifetime of methane in the atmosphere (230). The oxidation of methane results in the loss of 0.22 mol of OH radical per mol of methane destroyed in the atmosphere (379). The lifetime of methane in the atmosphere may be increased by as much as 20% by the year 2050 as a result of decreases in the concentration of OH radicals if methane emissions continue at current rates (230). On the other hand, decreases in the rate of methane emissions may have accelerating effects on atmospheric oxidation rates for this compound because of the increases of the concentrations of OH radical that would result. About 1.15 mol of ozone and 0.82 mol of CO are produced per mol of methane oxidized (379), and methane oxidation is a major source of atmospheric water (75, 119, 306, 379, 392).

Sources and Sinks of Atmospheric Methane

The total sources of atmospheric methane have been estimated at 520 Tg $year^{-1}$ (75, 139, 230, 290). The lifetime of this gas in the atmosphere is approximately 8 to 12 years (408). An amount equal to approximately 90% of the annual emissions (450 Tg $year^{-1}$) is oxidized through photochemical reactions initiated by OH radicals in the troposphere, and a smaller but significant amount (approximately 10 Tg $year^{-1}$) is lost by microbiological oxidation in soils (75, 139, 391). The net annual increase in atmospheric methane concentrations is estimated at 40 Tg $year^{-1}$. A 10% reduction in emissions of methane may stabilize its current concentration in the atmosphere (179, 230). Therefore, it is important to define the sources and sinks of atmospheric methane to determine which steps have practical value for reducing the global warming effects of this gas.

The increases in atmospheric methane concentrations described for the last 300 years are believed to result primarily from human activities (agricultural and industrial emissions), whereas preindustrial sources of methane were primarily natural wetlands with minor contributions from ruminants, fires, oceans, and insects (179, 230, 290, 316–318, 408). Although the total annual production of methane to the atmosphere and its rate of oxidation in the atmosphere can be estimated with reasonable accuracy (75, 139), there are significant uncertainties in the measurements of emissions from individual sources (321). Estimates of methane budgets including terms for global production, oxidation, and atmospheric emissions have recently been reviewed by Cicerone et al. (75, 76), Lelieveld et al. (230), Reeburgh et al. (318), and Bartlett and Harriss (31).

The studies reviewed below have been selected to demonstrate the roles of methane-utilizing microbes in the regulation of methane emissions to the atmosphere and to illustrate the environmental factors that affect the ecology of methanotrophs. The amount of methane that escapes to the atmosphere is the difference between the amount produced from the various sources and the amount consumed by methanotrophs and anaerobic methane-oxidizing bacteria. Some methane is consumed from the atmosphere by methanotrophs in soils of forests, grasslands, and other unsaturated soils, which are the major terrestrial sinks for atmospheric methane. The following descriptions of the sources and sinks serve to illustrate the interactions between microbes, plants, and physical factors that influence the rates of production and oxidation of methane in different ecosystems.

Methane production and oxidation in tundra and other wetlands. There are considerable differences in emission rates from different types of wetlands, and extrapolations from one wetland area or type to another may lead to considerable errors in estimates of emission rates. Tenfold or more year-to-year variations at a specific site have been observed, and subsites within a habitat gave more than 35-fold differences in emissions in some cases (28, 31, 89, 90, 111, 164, 252). Integrated eddy correlation measurements of methane fluxes from meteorological towers or aircraft are believed to yield the most reliable estimates of fluxes from an extended area (117).

The ecology of wetlands is complex. Wetlands are characterized as environments with standing water for all or part of the year. They are concentrated in the northern hemisphere north of 50°N and in the tropics where rates of precipitation are high (30, 31, 252). Precipitation rates in far northern latitudes are relatively low, and wetlands there are due largely to the presence of permafrost in soils, which prevents soil drainage. These environments are extensive (approximately 9×10^{12} m^2) and contain large amounts of stored carbon, and vegetation is limited (30, 31, 318). Further south, wetlands are more diverse and trees are present. Bogs or peat-producing wetlands are acidic and nutrient poor, while fens have surface water and are relatively nutrient rich.

Wetlands are considered to be the major source of atmospheric methane (about 115 Tg or 21% of the total atmospheric methane budget) (75, 90). Tropical wetlands (20°N to 30°S) have been estimated by Bartlett and Harriss (31) to account for 60% of the total wetland emission (66 Tg $year^{-1}$), while northern wetlands (north of 45°N) were calculated to release 38 Tg $year^{-1}$ (34% of the total) and subtropical and temperate wetlands release only 5 Tg $year^{-1}$.

The water depth, soil water content, type of vegetation, temperature, and other characteristics affect methane production and oxidation in wetlands. The major influences on methane emissions in tundra soils are vegetation and soil moisture content (207, 209–211, 384).

Methane transport through plant tissues often exceeds diffusion and ebullitive fluxes in vegetated wetlands and rice paddies (75, 92–94, 148, 211, 249, 276, 277, 340, 341, 345). Plant-mediated transport bypasses the soil surface oxidation of methane by aerobic methanotrophic bacteria, and plant trans-

port of methane has been estimated to account for 50 to 95% of the methane fluxes from wetlands (69, 76, 77, 92, 345, 348, 413). The influence of vegetation on methane fluxes is well illustrated by studies of methane emission from tundra soils (384). Very little methane is present in the upper 10 cm of saturated soils, and its concentration increases rapidly with depth to 40 cm below the surface (384). In environments where soils were saturated with water and were anaerobic except at the surface, vegetation biomass had the greatest effect on methane fluxes (384). Removal of plants reduced methane emissions to a small fraction of the flux rates observed in control sites. In sites where vascular plant populations were low, little methane escaped to the atmosphere, indicating that surface methanotrophs consumed most of the methane in the absence of plants. Bubier (57) found that bryophytes were better predictors of methane fluxes than were vascular plants. The presence of these bryophytes indicate that the long-term average position of the water table is favorable for methane production while shrub cover indicates dry conditions and low methane fluxes. Wagatsuma et al. (400) have shown that flooded soil microcosms planted with barnyard grass, Manchurian wild rice, early water grass, and reeds had decreased levels of methane within the soils whereas removal of plant tops below the water surface cause marked increases in methane concentrations in soil.

Wetland plants also provide carbon for methanogenesis and oxygen for decomposition. Reeburgh et al. (318) estimated that 30% of the methane produced was oxidized in high-latitude wetlands that contained mosses and plants. It was proposed that oxygen transport to the roots of plants increased methane oxidation rates. Epp and Chanton (124) and King et al. (212) observed rapid methane oxidation by the roots of several plant species from the Florida Everglades. Wagatsuma et al. (400) also demonstrated that the decreases of methane concentrations in soil, increases in nitrogen concentrations, and increases in E_h values in flooded soils were correlated with increasing hygrophyte plant densities. These changes were ascribed to gas exchange between the atmosphere and soils mediated by the plants. Reeds were particularly effective in transporting gases from the atmosphere to soils (400). Water lilies transport oxygen to rhizomes, which contain atmospheric levels of oxygen although they are surrounded by anoxic mud (93, 94). Methane also diffuses into the rhizomes and is transported through the plants to the atmosphere. About 46% of the methane lost to the atmosphere from Duck Lake, Mich., is transported through the leaves of water lilies (92).

The foregoing observations suggested that methanotrophs associated with plant root tissues, the rhizosphere, and plant surfaces played significant roles in methane oxidation in sediments and water. These observations also implicate plants as major pipelines for soil methane release to the atmosphere. Clearly, associations between plants and methanotrophs will be important determinants of methane fluxes.

Moore and Knowles (264) studied the effect of water table levels on methane and carbon dioxide emissions from three peatland soils in laboratory columns packed to a depth of 75 cm with surface materials (0 to 30 cm) from a fen, a bog, and a swamp. The soils ranged in pH from 3.7 to 4.9. The experiments were performed at 19 to 23°C, and the columns were initially inundated with water containing 10 mg of dissolved carbon per liter prepared by immersing tree leaves in water for 1 week to provide an influx of nutrients into the columns to mimic the natural nutrient sources. They observed that the methane evolution rates decreased exponentially as the water table was lowered from 10 cm above to 70 cm below the surface of the peat layers in the columns. The molar ratios of carbon dioxide evolved to methane evolved increased from 4 to 173 under different inundation conditions to >2,500 when the water table depth was 70 cm below the soil surface. The fen soils showed the highest rates of methane flux (28 mg of CH_4 m^{-2} day^{-1}) when inundated.

The primary sources of methane in the Florida Everglades are wet prairies and sawgrass marshes (165). Methane oxidation consumed up to 91% of the methane produced in peat soils, but little was consumed in marl soils (215). The wetter sites supported higher methane fluxes, and the fluxes dropped to low values when the water table dropped below the soil surface. When the soils of the Great Dismal Swamp dried, they ceased methane production and consumed atmospheric methane (165).

In every wetland studied by Crill et al. (90), the degree of inundation of a habitat by water was the dominant factor that correlated with methane flux. In moist tundra, where soils are not water saturated (no standing water), methane fluxes are low or negative, soil moisture, and temperature accounted for variability between sites (384, 410, 411). Methane emissions were described by the equation flux = $-53 + 10.8T + 0.04M$, where T is the soil temperature (°C) and M is the soil moisture (percent dry weight) (384). Methane production in tundra without standing water is limited because the oxidation-reduction potential is above that which allows methane production by methanogens and increased methane oxidation also reduces flux (209, 210). Flooded areas including beaver ponds, swamps, and small lakes are small methane sources. For both arctic and boreal regions, methane fluxes from dry soils were less than 10% of those observed for saturated soils. Roslev and King (324) observed that water table variations had a dramatic effect on methane oxidation and emissions from a shallow freshwater marsh near Walpole, Maine. The mean annual rate for methane oxidation in 1993 was 43% of the 22.2-g-m^{-2} methane production. In the spring and early summer, when the peat was submerged, 15 to 32% of the methane produced was oxidized, whereas in late summer and fall, the exposed peat consumed 48 to 76% of the methane produced. The availability of oxygen had a much greater effect than the temperature at the surface of the peat at this site.

In dry arctic soils and in the drier soils of the northern temperate and boreal wetlands that are invaded by shrubs and trees, methane is not produced and atmospheric methane is consistently consumed (31, 42, 89, 207, 209, 318, 408–411). These studies indicate that increased oxygen penetration and more rapid transport of methane to soil microbes in soils of low water content account for the increased rates of methane oxidation (318). Gas-phase methane diffusion is 10^4 times faster than is aqueous diffusion (408, 412).

Rice paddies. It has been estimated that methane production in flooded rice paddies is approximately 575 Tg $year^{-1}$ but that the amount escaping to the atmosphere is only about 100 Tg $year^{-1}$ (318). Measurements in flooded rice fields indicated that 80% of the methane produced was oxidized at the soil surface (84), although some estimates are lower (318). Fertilization of the water layer on top of the soil reversibly inhibited oxidation of methane, because ammonia and nitrate inhibit methane oxidation by methanotrophs (33, 84). The methane fluxes from vegetated paddy fields are much higher than from unvegetated fields (76, 77, 181). Most of the methane that escaped to the atmosphere in rice fields was transported through plants (339–342, 348). Mariko et al. (249) and Nouchi et al. (277) presented evidence that rice plants absorb methane from soil water surrounding the roots and that the methane volatilizes in the root cortex, is transported through intercellular spaces and aerenchyma, and is released through micro-

pores in the leaf sheaths. Thus, the internal air spaces of the plants acted as a ventilation system for the transport of methane from the soil water, where it occurred at 2,900 times the concentration found in the air surrounding the plants to the atmosphere. Rice paddies have relatively low emission rates compared with wetlands containing other plants on an area-for-area comparison (318). The low rates of rice plant transport may be explained by the lower permeability of rice root and rhizome cortex tissues compared with some other wetland plants (340, 341). The rate of plant transport in rice paddies was dependent on the methane concentration of the soil pore water and the maturity of rice plants in paddy fields (249). Large plants transported much more methane than did immature plants.

Dissolved oxygen was shown to be depleted within the top 3.5 mm of flooded soil microcosms containing soil from rice fields without plants but was detected to at least 40 mm deep in microcosms planted with rice (137). Oxygen concentrations in soil were higher in the light than in the dark when rice plants were present, and incubation under nitrogen atmospheres increased methane emission rates. It has been estimated that 80 to 90% of the methane produced in rice paddy microcosms was oxidized in the rhizosphere (84, 137, 181, 340).

Lakes. Freshwater lakes account for only a small portion (5 Tg $year^{-1}$) of the total atmospheric methane budget (76). Rivers are considered an insignificant source (344). In freshwater lakes, as in several other natural systems, methane oxidation is most active at interfaces between oxic and anoxic zones (151, 158, 166, 327–329). The availability and rate of production of methane, as well as the availability of oxygen and nitrogen, determines the location and rates of methane oxidation (151, 158, 166, 207, 327–329). In stratified, eutrophic lakes, methane oxidation occurs near the bottom of the chemocline (metalimnion), where dissolved-oxygen levels are relatively low compared with levels in the epilimnion during summer stratification. Very little methane is found in the oxygenated epilimnion, and most of the methane produced in the anoxic sediments is stored in the hypolimnion, which is also devoid of oxygen, or is oxidized in the metalimnion (151, 166, 235, 327–329). During the fall turnover of Lake 227, Canada, 95% of the annual methane oxidation occurred, and this oxidation accounted for removal of 60% of the methane from the lake (328, 329). The remaining 40% of the methane production was lost to the atmosphere during turnover (328). In Lake Mendota, Madison, Wis., more of the methane produced in the stratified lake was oxidized (45% versus 11% for Lake 227) because of greater wind turbulence and mixing of oxic and anoxic waters at the metalimnion (125). This mixing may result in reduced emissions of methane to the atmosphere from larger lakes that are more susceptible to wind turbulence than are smaller shield lakes. In dimictic eutrophic lakes, controls on methane emissions are related to the rates of primary production, which supplies substrates for methanogenesis, the supply of oxygen, and the rate of diffusion of methane into the metalimnion. A reduction of phosphorus inputs would limit primary production and the supply of substrates for methanogenesis, whereas increased aeration during summer stratification would decrease methane storage and release during turnover. In oxygenated shallow waters of eutrophic lakes and in lakes that contain oxygen throughout the lake water column, methane oxidation occurs primarily at sediment surfaces and little escapes to the atmosphere (209, 221, 329).

In deep, permanently stratified lakes like Lake Kivu, Zaire, in which the methane source is derived from geochemical processes in the Earth, little methane is lost to the atmosphere because methane is oxidized as rapidly as it diffuses to an oxygenated part of the water column (191).

It has been difficult to measure the contribution of methanotrophs to the methane fluxes when methanogenesis and methane oxidation occur simultaneously. Oremland et al. (291) used methyl fluoride to inhibit methane oxidation and observed increases in methane emission rates from sediments. They estimated that methanotrophic bacteria consumed more than 90% of the methane that was potentially available at some sites.

Oceans and hypersaline lakes and ponds. Methane is slightly supersaturated with respect to concentrations in seawater in equilibrium with the atmosphere. The highest concentrations are found in near-surface waters, although oceans are a minor source of atmospheric methane (75, 404, 408), contributing only 1.9% of the atmospheric methane budget. The subsurface maxima imply that in situ production processes that are not well defined are methane sources. It is possible that anaerobic niches in fish intestines, fecal pellets, and decaying plankton are sources (289). The efflux to the atmosphere is controlled by methane oxidation.

The reasons for the low rates of sediment methane production in most parts of the open ocean include the distribution of oxygen into sediments, low fertility (hence low primary productivity), and oxidation of organic carbon before it reaches the sediments of the deep ocean.

In parts of the sea, the sea bottom is made up of organic-rich sediments due to inputs from rivers, coastal sediments, oil wells, upwelling, or natural sources of nutrients including subsurface natural gas and hydrocarbon deposits. The data of Scranton and McShane (344) indicated that oil wells and drilling rigs are a minor source of methane. Near the coast of the Netherlands in the North Sea, methane concentrations in the plume of the Rhine were 120 times those found in open areas of the North Sea, which were in equilibrium with the atmosphere (344). The dominant sources of this methane were dissolved methane and methanogenesis driven by organic carbon in the Rhine and Scheldt rivers, where the methane concentrations were as high as 5 μM. The oxidation of organic matter resulted in anoxic conditions in sediments, which may have caused them to become methane sources. Methane oxidation rates were extremely low. The highest rates observed were 5 nmol $liter^{-1}$ day^{-1}, and most of the methane was lost to the atmosphere (344). However, this methane source is a very small part of the global atmospheric budget.

Cold gas seeps from subsurface methane and hydrocarbon deposits occur in a variety of marine environments including the Gulf of Mexico; the Kattegat strait between Denmark and Sweden; the Northern Continental shelf of Norway; off Baffin Island, Canada; and along the coasts of Alaska and Japan (179, 199, 205, 224). Off the Louisiana coast, seeps cover a vast area (hundreds of square kilometers) at relatively shallow depths. Both hydrocarbons and methane are present in the seeps. Bacterial mats, tube worms, mussels, and coral reefs are found in the vicinity of these seeps (103, 183, 192, 198, 205). Little is known about the free-living microbes in the water columns above the seeps. LaRock et al. (224) have shown that the bacterioplankton in waters above the seeps may have very high growth rates (generation times of about 1.1 h) as measured by phosphate uptake rates. Marine snow is ubiquitous to the Louisiana seep regions, and these bacteria may provide a food resource that may spread for hundreds of kilometers (4, 183). It is believed that methane may serve as the primary carbon and energy source for the microbial assemblages that form marine snow in waters above and near the seeps. The methane concentrations in the seeps are in the millimolar range (241).

LaRock et al. (224) extracted RNA from bacterioplankton filtered from the water above the seeps, probed it with phylogenetic signature probes for type I and type II methanotrophs, and observed hybridization with both probes.

Marine waters near hydrothermal vents located at midoceanic spreading centers at depths of 2,000 to 3,000 m have elevated levels of methane (238, 406) and are the sites of aerobic methane oxidation (109, 110). Methane oxidation rates by water samples were stimulated 21 to 62% by increasing the hydrostatic pressure to in situ levels (200 atm [2.03×10^7 Pa]). Methane emitted from continental shelf seeps and deep-sea vents is oxidized by aerobic microbes surrounding the seeps or existing as intracellular symbionts of mussels (64, 66, 70, 129, 250, 251). These symbiotic relationships and others are described in detail in the section on associations of methanotrophs with other bacteria, invertebrates, and wetland plants (below).

In the open ocean, methane concentrations and rates of methane oxidation are low. In the Sargasso Sea, methane concentrations decreased only slightly in the top 500 m and microbial oxidation of methane was very slow compared with that in more productive waters. The turnover time for methane was 5 to >100 years. Very low rates of methane oxidation have also been observed for the Bering Sea and the Caribbean Sea (403).

Anaerobic methane oxidation in anoxic marine waters and sediments is believed to be an important sink, consuming 20 to 200 Tg of methane per year (219, 312–317). Anaerobic methane oxidation has been examined in anoxic marine shelf sediments, which are rich in organic compounds (219, 251, 312–315, 403). In anoxic marine basins containing organic matter, methane is produced by methanogenesis in the sediments (343, 403). Methane concentrations are very low in anoxic marine sediments that contain sulfate, and they increase dramatically in deeper sediments that do not contain sulfate (219). Kosiur and Warford (219) incubated anoxic sediments from the Santa Barbara Basin and observed that methane was oxidized within and below the sulfate-reducing zone provided that sulfate was initially present.

In the Cariaco Basin, the deeper waters are anoxic and an oxic/anoxic interface is found between 260 and 300 m deep (403). Methane accumulates to high concentrations below the chemocline. Aerobic methane oxidation occurs at the oxic/anoxic interface, but much higher rates of oxidation were found in the deeper anoxic water (403).

The Black Sea is the largest surface water reservoir of dissolved methane (96 Tg) and the largest anoxic saltwater basin (317). Methane concentrations were 10 nM in the upper 100 m, increased to 11 mM at 550 m deep, and were uniform to the sediment surface (317). The dominant methane sink was anaerobic methane oxidation (317). Methane consumption in the absence of oxygen in the Black Sea occurred below 100 m and into the sediments. Higher in the water column, aerobic methane oxidation was the dominant sink but rates were 100-fold lower than in anoxic waters. Anaerobic oxidation rates in sediments were as high as 100 $\mu M\ m^{-2}\ year^{-1}$. Losses of methane to the atmosphere were estimated to be 4.1×10^9 mol $year^{-1}$ for the entire basin.

There is also evidence for anaerobic methane oxidation in hypersaline alkaline lakes including Big Soda Lake, Nev., and Mono Lake, Calif. (190, 291), and sediments of freshwater lakes where sulfate inputs are significant.

Soils and sediments. Aerobic soils and sediments serve as biofilters for methane produced in anoxic soils or anoxic sediments, and some soils serve as sinks for atmospheric methane (36, 164, 165, 203, 216, 342). The current temperate soil sink is estimated at 20 Tg $year^{-1}$ (282). Most unsaturated soils consume atmospheric methane (42, 203, 212, 216, 282). The rate of methane oxidation varies with soil water content, land use and ammonium inputs (33, 37, 89, 115, 203, 213, 214, 271, 410–412). Consumption of atmospheric methane has been demonstrated in coniferous and deciduous forest soils (89, 201, 428), agricultural soils, grasslands (16, 42, 202, 257), and tundra soils (410, 411). The amount of atmospheric methane consumed by oxic soils has been estimated at 40 to 60 Tg per year (75, 318, 342). This amount is approximately equal to the annual increase in atmospheric methane during the past century. Methane uptake was measured as 0.5 to 5.5 kg of CH_4 $ha^{-1}\ year^{-1}$ for deciduous forest soils (201), 0.5 to 2.9 kg ha^{-1} $year^{-1}$ for undisturbed tropical forests (203, 318), and 4.6 kg $ha^{-1}\ year^{-1}$ for subtropical woodlands.

Atmospheric methane uptake is decreased after fertilization of soils with nitrogen, conversion of grasslands to croplands, tillage, and clearing of forest lands (63, 203, 216, 265, 282, 373). The conversion of forests and grasslands to croplands results in a reduction in methane consumption in these ecosystems of 1.5 to 7 Tg $year^{-1}$ (282). Fertilization of a slash pine plantation in Florida lowered the methane uptake rate by 5- to 20-fold (2, 63). The subsurface maxima for methane oxidation in subarctic mixed-hardwood forest soils coincided with low levels of nitrate and ammonia (2).

Estimates of methane oxidation in wheat fields were 75% less than uptake in a nearby undisturbed grassland site (282). Fertilization of grasslands with 22 kg of ammonium nitrate per acre for 15 years reduced methane uptake considerably (265). Adamsen and King (2) observed that the addition of 1 μmol of ammonium salts or sodium nitrate per g of soil inhibited methane oxidation immediately by 61 to 93%. This may be attributed to a salt effect on methane oxidation.

Schollenberger (338) observed that natural-gas leaks from pipelines resulted in higher soil nitrogen and organic matter contents than are found in normal soils. Davis et al. (107, 108) isolated methane-oxidizing bacteria capable of fixing atmospheric nitrogen from a variety of soils and attributed the high organic nitrogen content in soils exposed to leakage from pipeline or natural-gas seeps to the activities of these bacteria. Most of the methane that escapes from buried pipelines in all but the largest leaks is oxidized in soils adjacent to the leaks (178).

Deserts. Deserts constitute 20% of the Earth's total land surface. They contain little carbon to support methanogenesis, and they are aerobic to depths of several meters during dry cycles. Desert soils consume atmospheric methane (371). The global sink for atmospheric methane by desert soils was estimated to be approximately 7 Tg per year.

Mojave desert soils oxidized atmospheric methane at rates of approximately 62.5 $\mu M\ m^{-2}\ day^{-1}$, and rates as high as 274 $\mu M\ m^{-2}\ day^{-1}$ were measured in flux chambers at four different sites (371). Methane consumption occurred to depths of at least 2 m but was most active within the top 0.75 m of the soils. The rate of methane oxidation decreased dramatically after rainfall because of the decreased oxygen content of the soils, and it increased rapidly within hours as the soils drained. When they continued to dry, oxidation rates decreased dramatically. These results indicate that the methane-oxidizing microbes are limited by the available moisture and are able to recover within a few hours after precipitation. These results also imply that the methanotrophs in this environment survive periods of drying in an inactive state and can recover their ability to oxidize methane rapidly when moisture becomes available.

Landfills. Landfills are estimated to produce 30 to 70 Tg of

methane to the atmosphere per year. On the other hand, very high rates of methane oxidation (up to 45 g or 3 mol m^{-2} day^{-1}) have been observed in landfill cover soils by Whalen et al. (412). These are the highest rates of methane oxidation observed in natural soils. Kightley et al. (208) observed that coarse sandy landfill soil microcosms permeated with methane for 6 months oxidized 10.4 mol of CH_4 m^{-1} day^{-1}. Whalen et al. (412) estimated that methane oxidation consumed approximately 50% of methane production (35 Tg $year^{-1}$) in landfills in the United States. Moreover, the landfill soils oxidized methane with first-order reaction rate constants at concentrations from as low as 1 ppm ($k = -0.54$ h^{-1}). The first-order reaction rate constant at 10^4 ppm was -2.37 h^{-1}. Methane oxidation rates in these soils were also influenced by water concentrations.

The rate of methane oxidation in waterlogged landfill soil (41% by weight of water) was 6.1 mg per day, which was about the same as rates observed in oxic freshwater sediments, and was 116 mg per day in soils with 11% water content. Low soil moisture content also limits methane oxidation in landfill soils. Adamsen and King (2) observed that decreases of the water content to 5% resulted in dramatic decreases in methane oxidation rates.

Other sources of atmospheric methane. Ruminants, coal mining, oil and gas recovery, and gas transport are significant sources of methane. However, with the exception of buried gas pipelines (318), methane consumption at these sources has rarely been measured.

The foregoing discussion points out the difficulties involved in modeling the factors that control atmospheric methane emissions. In general, it appears that oxidation of methane occurs to globally significant extents both aerobically in soils and waters and anaerobically in sulfate-rich sediments and anoxic saline waters. In soils and sediments, low methane fluxes and atmospheric methane consumption correlate with low water content and no chemical fertilization. High methane fluxes and inhibition of atmospheric methane oxidation are seen in response to high water contents and application of chemical fertilizers. Vegetation in submerged and nonsubmerged soils can both increase and decrease methane flux by providing an easy conduit for the transport of soil methane to the atmosphere and by oxygenating the rhizosphere and stimulating rhizosphere methanotrophs. Recovery of methane from landfills and reductions in freshwater eutrophication would cause a small reduction in atmospheric methane emissions. Soil oxidation of atmospheric methane would be increased by less use of nitrogenous fertilizers and pesticides, and a reduction in the number of sources that vent methane directly to the atmosphere (ruminants, coal mining, oil recovery, and natural gas leaks) would be beneficial. Interestingly, the destruction of temperate wetlands may have reduced methane emissions by a small degree, but wetlands that have been drained did not account for significant levels of methane emissions.

ECOLOGY OF METHANOTROPHIC BACTERIA

Detection of Methanotrophic Bacteria and Their Activities in Natural Samples

Nearly all samples taken from muds, swamps, rivers, rice paddies, oceans, ponds, soils from meadows, deciduous woods, streams, sewage sludge, and several other environments contained methanotrophic bacteria (87, 151, 155, 158, 170, 172, 173, 180, 346, 347, 370, 381, 417). Methanotrophic bacteria are also known to be endosymbionts of mussels, and there is increasing evidence that they are closely associated with plants in several aquatic sediments and pond waters. Some hypersaline environments do not appear to contain aerobic methanotrophs (83), although anaerobic methane oxidation occurs in the water columns of hypersaline alkaline soda lakes (190, 291).

Well-characterized methanotrophic bacteria are obligate aerobes. Some, including the type II and type X methanotrophs, are capable of fixing dinitrogen (48, 155, 417). When forced to fix nitrogen in environments depleted of combined nitrogen (some freshwater lakes, peat bogs, etc.), they are found in environments low in dissolved oxygen (158, 166, 327, 381).

Generally, the number of methanotrophs isolated as CFU from pure cultures is a small fraction of the viable population and the fraction of cells recovered from environmental samples is also believed to be a small fraction of those present (17, 41, 151, 158, 381). The numbers of methanotrophs detected in soils, sediments, and waters ranged between 10^3 and 10^6/g when viable-count procedures were used (170–172). The physiological types of methanotrophs isolated from samples may reflect the conditions used for enrichments and isolation attempts more than the dominant organisms in the original population (17, 152, 158, 170, 415, 417). As discussed below, some populations of methanotrophs in soils and some other natural environments appear to have characteristics different from those available in pure culture collections. Therefore, it has been necessary to use indirect methods to measure population sizes and to characterize the methanotrophs present in different habitats. Methane oxidation rates determined by methane consumption with a gas chromatograph or by the oxidation of radiolabelled methane to carbon dioxide have been used to measure the physiological activity of the methane utilizers (166, 297, 327–329).

The use of fluorescent antibodies prepared against killed cells from pure cultures is a potentially effective means of identifying and enumerating methanotrophs and other bacteria without culturing them (1, 40, 50, 140, 319). However, these techniques require that the organisms used to prepare antisera belong to all the serotypes present in the habitats under study. Gal'chenko et al. (140) used indirect immunofluorescence with antibodies prepared against 14 methanotrophs isolated from soils and freshwaters to determine the numbers and species of methanotrophs in sediments and waters of the Black Sea. The numbers of methanotrophs detected were larger by 3 orders of magnitude than the numbers detected by viable-count procedures, demonstrating that in some environments, antibodies can detect methanotrophs more efficiently than can methods dependent on recovery of viable cells. Reed and Dugan (319) found that the recovery of *Methylosinus trichosporium* cells from sediments was very low (1 to 2% of the cells added to the sediments).

Unculturable microbes that have been proven viable and/or detectable under the microscope but that cannot be cultured can be detected with nucleic acid probes or by sequencing genes amplified by PCR or cloned directly from environmental samples (14, 15, 135, 144, 281, 285, 292). These methods are useful for identification of taxa and for determination of the phylogenetic positions of microbes whose rRNAs have been recovered from environmental samples and could not otherwise be characterized. The sequences and the strength of hybridization reactions with specific probes can be used to infer population sizes and physiological activities (14, 15, 135, 144, 237, 281). The probes used range from oligonucleotides or cloned DNA fragments which detect genes encoding enzymes unique to different physiological groups of bacteria to phylogenetic signature probes which are complementary to 16S

rRNA sequences that are conserved within a particular group and are unique to that group of bacteria. For the methanotrophs, the genes encoding the sMMO components and MDH have been used to identify methanotrophs within natural communities, as have phylogenetic signature probes that hybridize to 16S rRNAs of type I or type II methanotrophs. The sequence of a pMMO component is now known, and it appears to be highly conserved but is similar to the sequence of AMO found in nitrifying bacteria. It is important to realize that all hybridization-based detection strategies (gene probes, oligonucleotide probes, and PCR) are subject to the same pitfalls. Not all organisms may have conserved all genes similarly, and genes obtained from cultured organisms may not be sufficiently similar in sequence to genes in the environment, because the two populations were subjected to different selection pressures. For example, the uniqueness of a sequence within the 16S rRNAs of a group of microbes is known only to the extent of the sequences available in the different data banks. Uncultured methanotrophs may not share the same conserved sequences that have been identified in type I and type II methanotrophs, and the phylogenetic signature probes would not detect their 16S rRNAs when they are extracted from environmental samples.

Analysis of the fatty acids in phospholipids extracted from environmental samples is useful for measuring changes in community structure and physiological stress within a microbial community (273, 274). This approach is particularly useful for the detection of populations of type I and type II methanotrophs because of the unusual fatty acids they contain. The signature lipids of the methanotrophs described have been used to measure changes in the methanotrophic bacterial community during exposure of soils to methane and in bioreactors (273, 274).

A great deal of information has been obtained about the ecology of methanotrophs by the different techniques. The application of the various techniques to ecological studies of methane-utilizing bacteria and information on the properties of these bacteria gained from studies of the kinetics of methane utilization in different habitats are described below.

Methanotrophic Bacteria in Soils, Sediments, and Water

No pure cultures of methanotrophic bacteria show kinetic properties that explain methane oxidation at the low methane concentrations found in soils in which the atmosphere is the primary source of methane (34, 35, 82). The relationships between K_s and V_{max} values of known methanotrophs and values for environmental samples as well as populations of methanotrophs in different environments have been measured (34, 35, 82). Convincing arguments have been presented that the kinetic values obtained with pure cultures would not permit methanotrophs in environmental samples exposed to atmospheric methane to sustain the oxidation rates required for the maintenance and growth of indigenous populations of obligate methanotrophs. In soils in which the only source of methane is atmospheric gases, the methane concentration dissolved in soil waters is 2.5 nM at 20°C. In soils, sediments, and eutrophic lakes where methanogens in anaerobic regions supply methane that is oxidized in the top layers of the sediment or in the water column, the methane concentrations are in the micromolar to millimolar range.

The K_m values for methane measured with purified MMO is 3 μM, and the V_{max} has been reported to be 56 nmol mg of protein^{-1} min^{-1} (134). The K_s values for methane oxidation were determined to be between 0.8 and 9.3 μM for pure cultures of methanotrophs (33, 195, 196, 209, 411, 412). The differences in these values can be accounted for by the methods used. When diffusion did not limit methane oxidation, the K_s values reported were within a range of 1.0 to 2.0 μM for pure cultures (195, 196). Growth conditions may also affect the K_s values, because the pMMO appears to have a higher affinity for methane and oxygen than the sMMO does (33, 99, 100, 158, 210).

The K_s values for methane oxidation in soils exposed to subsurface sources of methane range from 2.2 to 2.5 μM for landfill, peat, and wetland soils (212, 248, 412, 428) and still higher for soils enriched by exposure to methane in laboratory studies (35, 36). The apparent K_m values for methane oxidation by lakewater samples are generally in the range of 5 to 10 μM (58, 166, 210, 235, 320) and were approximately 0.5 μM in marine water samples from the Southern Bight of the North Sea, which had relatively low methane concentrations. The half-saturation constants for methane oxidation by soils exposed to atmospheric methane only (cultivated cambisol, forest cambisol, meadow cambisol) ranged from 22 to 45 ppmv (32 to 66 nM in the aqueous phase). Methane oxidation by soils that oxidized atmospheric methane was a first-order reaction until the methane concentration fell below 0.5 ppmv. The threshold concentrations at which methane was not oxidized (as low as 0.04 to 0.06 nM) were much lower for aerobic forest and grassland soils than for soils with subsurface sources of methane, like landfill soils, and lakes, where the threshold values were in the range of 50 to 150 nM (335, 412).

The vertical profiles of the maxima of methane oxidation in soils studied by Bender and Conrad (36, 37) correlated with populations of methane-utilizing methanotrophs in stratified, undisturbed soil and sediment cores when the naturally occurring methane concentrations were 1,000 ppmv or higher. This condition existed in soils and sediments with subsurface sources of methane. In oxic soils exposed only to atmospheric concentrations of methane, there was no correlation between the distribution of the numbers of culturable methanotrophic bacteria and the distribution of methane oxidation activity in vertical profiles.

Populations of methane-oxidizing microorganisms were correlated with the methane content in soil when methane composed up to 50% by volume of the gas (37, 152, 158, 247, 381). Bender and Conrad (36, 37) measured methane oxidation rates and populations of methanotrophs by using most-probable-number techniques in oxic freshwater lake sediments and a variety of soils. At high methane mixing ratios found in sediments (1,000 ppmv or higher), the number of methanotrophic bacteria correlated with the methane oxidation rates in samples taken at different points in the soil column. In soils exposed to lower methane concentrations (2 ppmv or lower), maximum methane oxidation rates occurred at depths greater than the depths at which the maximum number of methanotrophs were observed by the most-probable-number techniques used. When soils with low affinity constants for methane oxidation were exposed to high levels of methane, the K_m values for methane oxidation increased to values equivalent to those for pure cultures of methanotrophs (50,000 ppmv) (37). These authors interpreted the results to indicate the presence of two different populations of methanotrophs in soils exposed to different methane concentrations. One population, which remains to be characterized, hypothetically oxidized methane at low (atmospheric) concentrations and had a high affinity for methane, whereas soils exposed to higher concentrations of methane contained populations of methanotrophs with affinity constants for methane similar to those observed in pure cultures of known methanotrophic bacteria (greater than 1 μM) (36, 37, 257, 410). Alternatively, the classical methanotrophic

bacteria may express a high-affinity methane-oxidizing system when exposed to low concentrations of methane. The oxidation of methane in these systems may be a fortuitous reaction catalyzed by enzymes of some undefined organism.

Kightley et al. (208) studied methane oxidation in microcosms containing different soils that had been exposed to methane for up to 6 months. Porous coarse sandy soil developed the greatest capacity for methane oxidation (10.4 mol of methane m^{-2} day^{-1}). The highest rates of methane oxidation occurred when the vertical profiles of methane and oxygen overlapped. These microcosms had a low affinity for methane (K_{app}, 31.7 nM) but a high V_{max} (998 nmol g of $soil^{-1}$ h^{-1}). On the other hand, microcosms exposed to nitrogen rather than methane had relatively low methane oxidation capacities (V_{max}, 258 nmol g of $soil^{-1}$ h^{-1}) but had high affinities for methane (K_{app}, 1.6 nM). These authors suggested that facultative methanotrophs with a high affinity for methane were responsible for its oxidation in microcosms exposed to nitrogen and oxygen.

Bender and Conrad (37) determined the effects of methane concentrations and several different soil conditions on the induction of methane oxidation activity and increases in the populations of methanotrophic bacteria as measured by a most-probable-number technique (34–37). They observed that four different soils exposed to 1000 to 200,000 ppmv of methane increased the methane oxidation potential. The optimum conditions for growth of methanotrophic bacteria and induction of methane oxidation activity were water contents of 20 to 35%, temperatures of 25 to 35°C, pH values of 7.0 to 7.65, ammonia concentrations between 12 and 61 mM, and copper concentrations of less than 4.3 mM.

Increases in the populations of methanotrophs were detectable only at methane concentrations exceeding 7,000 ppmv (37). At a methane mixing ratio of 200,000 ppmv, a 100-fold increase in methane oxidation by soils and 10- to 100-fold increases in populations of methanotrophs were observed. At 7,000 ppmv of methane, the increase in methane oxidation rates at steady state was as high as 10-fold, although increases in populations of methanotrophs were much smaller than at concentrations of 100,000 ppmv or greater.

Although 100 ppmv of methane (0.14 μM in the aqueous phase) was calculated as being sufficient to account for increases in methanotroph populations of approximately 10^4 cells per g of soil, this number of bacteria was smaller by 2 orders of magnitude than the indigenous populations of methanotrophs detected, and a concentration of 7,000 ppmv was calculated to be required for a detectable increase over indigenous populations of approximately 10^6 cells per g of soil (37). Heyer et al. (172, 173) have also shown that high concentrations of methane are required for the germination of resting stages of methanotrophs.

Soils exposed to less than 100 ppmv of methane oxidized methane but did not show detectable increases of methane oxidation activity (37). These results may indicate that indigenous methane-oxidizing bacteria have high-affinity systems for the oxidation of methane (34–37, 82).

The results of Schnell and King (337) support the hypothesis that methanotrophs are responsible for atmospheric methane oxidation. They observed that methane oxidation in cores from forest soils exposed to 1.7 ppmv of methane for 10 to 12 weeks continued at constant rates whereas soils incubated with 0.03 ppmv of methane lost the capacity to oxidize methane. Methane uptake rates did not increase in soils exposed to 17 and 170 ppmv over those in soils exposed to 1.7 ppmv. The rates of soil methane oxidation (measured at 1.7 ppmv) did increase after incubation of the soils at 1,000 ppmv methane, presumably as a result of growth of methanotrophs, but decreased rapidly when these soils were reexposed to 1.7 ppmv.

Several results suggested to these authors that atmospheric methane was the major source of carbon and energy for the growth of soil methanotrophs. The fractions of methane carbon oxidized to carbon dioxide and the methane carbon incorporated into cell material by soils exposed to atmospheric methane was approximately the same as for pure cultures of methanotrophs (approximately 0.5). Hydrogen, methanol, formate, glucose, starch, and yeast extract did not cause increases in rates of methane oxidation when added to the soils. The soils used had low levels of ammonium, and additions of ammonium, nitrate, copper, and a variety of cations and anions did not increase the rates of atmospheric methane oxidation. Antibiotics (streptomycin, tetracycline, chloramphenicol, ampicillin, and the eukaryotic antibiotic cycloheximide) all caused rapid decreases in the rates of soil methane oxidation. These authors also measured rates of methane oxidation by *Methylosinus trichosporium* and *Methylobacter albus* at ambient concentrations and calculated that the rates of ambient methane oxidation by soils exposed to atmospheric levels of methane were equivalent to approximately 3.8×10^8 cells per g of soil, which seemed unrealistically high to them. They also noted that laboratory strains of methanotrophs would not survive in culture at 1.7 ppmv of methane under aerobic conditions (324). They suggested that the soil methanotrophs depended on methane as a source of carbon and energy to maintain the populations observed in soils that oxidize atmospheric methane and that these bacteria had higher affinities for methane than did the methanotrophs that had been characterized.

It is improbable that nitrifying bacteria which have lower affinities for methane (6.2 to 2,000 μM) than the K_m values reported for methanotrophs (33) are responsible for methane oxidation. The rates of methane oxidation by ammonia-oxidizing bacteria (0.6 to 1.9 mmol of methane oxidized g^{-1} h^{-1}) are much lower than those reported for methanotrophs (10 to 31 mmol of methane g^{-1} h^{-1} (33). Therefore, it seems improbable that a sufficient biomass of ammonia-oxidizing bacteria would be present in soils to account for the observed rates of methane oxidation at low mixing ratios (43, 336, 337).

Effects of Temperature and pH on Methane Oxidation by Methanotrophic Bacteria

Most methanotrophs available in pure culture are mesophiles, although *Methylococcus capsulatus* Bath and related strains are capable of growth at temperatures up to 50°C (20, 48, 49, 155) and psychrophilic methylotrophs have been described recently (287). Different soils exhibit different methane oxidation responses with respect to temperatures, indicating that populations of methanotrophs in nature also adapt to different temperatures. The optimum temperature for methane oxidation was 25°C in most peat soils, although oxidation occurred at 0 to 10°C and at 35°C (33, 37, 116, 209, 410, 411). Dunfield et al. (116) found that northern peatlands showed no marked adaptation of methanotrophs to low-temperature environments. The Q_{10} values were 1.4 to 2.1, and the activation energies for methane oxidation were 20 to 80 kJ mol^{-1}. The rates of methane oxidation at 0 to 5°C were 10 to 38% of the maximum rates observed at 25°C. On the other hand, Omel'chenko et al. (286, 287) isolated methanotrophs from acid soils in a bog hollow in the Arctic that had optimum growth temperatures of 10°C or lower. Three strains that were isolated and characterized all grew at 3.5°C. One culture failed to grow at or above 15°C. The growth rates were 0.25 day^{-1} or lower under optimal conditions. One strain, Z-0021, which

grew best between 3.5 and 10°C and less well at 15°C, was shown to contain high levels of hexulosephosphate synthase and lacked ribulose-1,5-diphosphate carboxylase and α-ketoglutarate dehydrogenase (286). The bacteria were cocci that occurred singly or in pairs. They resembled the moderate thermophile *Methylococcus capsulatus* in cell morphology and the presence of type I intracytoplasmic membranes (286). However, there was very low DNA homology with *M. capsulatus* Texas. These psychrophilic strains of methanotrophs also contained gas vesicles, particularly when they were exposed to temperatures of 20°C. Therefore, it can be concluded that some type I methanotrophs are psychrophiles. It will be interesting to determine if these strains are closely related to other type I methanotrophs by comparing 16S rRNA sequences.

The optimum pH values for methane oxidation and growth of methanotrophs in soils are generally consistent with the optimum pH values for the growth of most known methanotrophs in pure cultures (116). Borne et al. (42) observed that methane oxidation rates were similar in soils from pH 3.5 to 8.0, and Heyer and Suckow (174) reported oxidation in peat samples at pH values of 3.7 to 4.4. Dunfield et al. (116) demonstrated that the pH optimum for methane oxidation in acidic peats (pH 6.0 to 7.0) was at least 2.5 pH units higher than the pH in native peat (pH 3.5) from a Hudson Bay lowland. They concluded that the methane-oxidizing microfloras in these acidic environments were neutrophilic methanotrophs. This conclusion was also supported by the studies of Bender and Conrad (37). In some acidic peat samples, the rates of methane oxidation were only slightly influenced by pH at values between 4.0 and 6.0 and decreased sharply at values below and above this range, although oxidation still occurred below pH 4.0. The pH optima and minima appeared to be slightly lower in acidic than in alkaline peats and soils, and the rates of methane oxidation at pH 4 to 5 were higher in acidic peats (42, 116) than in the more alkaline soils. These results suggest that methanotrophic populations are only partially adapted to acidic environments.

There are no reports of methanotrophic bacteria that grow at pH values below 5.0. Despite numerous attempts, our group has failed to isolate or obtain enrichments for methanotrophic bacteria that would grow at pH values below 5.5 from acidic peat samples. However, Wolf and Hanson (422, 424, 425) reported the isolation of several yeast strains that oxidized methane after enrichment at a pH of 3.5 in a rich medium and isolation of colonies on a defined agar or other solid supports with a defined medium containing vitamins and amino acids at pH 5.5. The yeasts grew best at pH values below 6.0 and were shown to be facultative methanotrophs (424, 425). Saha and Chen (330) also reported that a strain of *Candida tropicalis* oxidized methane.

Effects of Fertilization with Nitrogenous Fertilizers and of Additions of Nitrification Inhibitors and Pesticides on Methane Oxidation by Methanotrophic Bacteria

Ammonium fertilizers inhibit the oxidation of atmospheric methane by cropland, grassland, forest soils, sediments, lakewater, and marine waters (33, 37, 43, 63, 213, 216, 265, 336, 373, 402) and in pure cultures of methanotrophs (288). Ammonia completely inhibited methane oxidation in sediment slurries from Lake Constance, Germany, at concentrations above 20 mM when the methane concentration was 21 μM. In sediment cores, ammonia concentrations below 4 mM in pore water caused no inhibition of methane oxidation whereas ammonia concentrations between 4 and 10 mM caused an approximately 30% reduction in the rate of methane oxidation. It has been suggested the NH_3 rather than NH_4^+ is the inhibitor of MMO (33, 257), although the effect of NH_3 inhibition of methane oxidation in soils may be more complex. Nitrite produced from the oxidation of ammonia by methanotrophs (33) has been found by King and his coworkers to cause a more permanent inhibition of methane oxidation than ammonia by an unknown mechanism (210, 213, 214). However, Willison et al. (418) found that the addition of ammonium fertilizers to grassland at the Rothamsted Experimental Station, Rothamsted, United Kingdom, for 138 years caused a significant decrease in atmospheric methane oxidation whereas the application of nitrate-N for the same period did not.

The ecological implications of ammonia, nitrate, and nitrite inhibition of methane oxidation are significant. The application of fertilizers and agricultural runoff undoubtedly restrict methane oxidation in agricultural areas (33, 209, 210, 214, 247, 259). The inhibition of methane oxidation persists for long periods (210, 214), and because of the large areas involved, the loss of atmospheric methane oxidation potential and that occurring in wetlands, rice paddies, and other areas receiving runoff is significant.

N-Serve is commonly added to soils to prevent losses of nitrogen through nitrification and denitrification. This compound inhibits ammonia-oxidizing bacteria and inhibits methane oxidation in pure cultures, soils, and sediments (33, 258, 331, 380, 381, 383). The herbicide bromoxynil and the insecticide methomyl also inhibited methane oxidation in soil slurries when they were added at concentrations of 50 mg liter^{-1} (380).

Distribution of Different Types of Methanotrophic Bacteria in Natural Habitats

Specific rates of methane oxidation by pure cultures and natural samples. Knowles (216a) has found the specific rate of methane oxidation is 0.2×10^{-15} mol per cell per h for *M. trichosporium* OB3b cells grown in AMS medium (417). The V_{max} for methane oxidation by pure cultures of methanotrophs is 10 to 31 mmol of CH_4 g^{-1} h^{-1} (33). Bender and Conrad (36) measured methane oxidation rates and the numbers of methanotrophic bacteria in soils by a most-probable-number MPN technique for samples taken from soil and sediment cores. In soils containing approximately 20% moisture, the specific rates of methane oxidation for various soils exposed to atmospheric concentrations of methane ranged between 0.2×10^{-15} and 1.5×10^{-15} mol of methane oxidized per viable cell per h whereas a soil exposed to high levels of methane (50,000 ppmv) had a specific rate of methane oxidation of 1.5×10^{-14} mol of methane oxidized per viable cell per h. The specific rates of methane oxidation for freshwater sediments was 9×10^{-14} mol of methane oxidized per viable cell per h. They related methane oxidation rates (V, in nanomoles per hour) at 100 ppmv methane or higher concentrations to the numbers (N) of methanotrophic bacteria according to the equation $\ln V = aN + b$, where $a = (0.144 \pm 0.03$ [standard deviation]$) \times 10^{-6}$ and $b = 0.14 \pm 0.8$ standard deviation; ($r^2 = 0.83$).

The mean value of V for a viable cell in the soils examined by these authors was approximately 0.3×10^{-15} mol/h. The agreement with the values given by Knowles for pure cultures was very good. The values for the soils measured by Bender and Conrad (34, 36, 37) assume that the viable-count methods detected nearly all of the methanotrophs in the soil samples.

Brusseau et al. (55) isolated RNA from a soil sample obtained from 0 to 10 cm below the surface of soils near the Kuparuk River on the Alaskan North Slope. After enrichment with methane and air for 1 month, the rates of methane oxidation in the soils increased from 0.1 to 5.0 mmol g^{-1} h^{-1}.

RNA was extracted from the soil microbes, and the 16S rRNA was separated from material that interfered with hybridization reactions by agarose gel electrophoresis and was Northern (RNA) blotted to nylon membranes. Hybridizations with probes complementary to unique sequences in the 16S rRNAs of type I and type II methanotrophs indicated that type II methanotrophs dominated in these enriched soils, although much smaller populations of type I methanotrophs were detected.

In low-temperature, slightly acidic tundra bog soils that were overlain with water, methane oxidation occurred in the water layer and within the moss layers at the boundary of the turf horizon. Immunofluorescence analysis with antisera prepared against pure cultures of methanotrophs and labeled with fluorescent compounds revealed the presence of several species of type I and type II methanotrophs throughout the thawed layer of the soils (394). The total number of methanotrophs detected varied between 1.5×10^6 and 2.3×10^7 cells g of soil^{-1}. They represented 1 to 23% of the total microbial population that was detected. The most abundant of the methanotrophs detected were members of the type I genera (61.6%), with fewer numbers of the genera *Methylosinus* and *Methylocystis* (38.4%), although there was considerable variation in the numbers of type I or type II methanotrophs and species within these groups in different soils and overlying waters.

When natural gas and air were passed through a soil column, the level of the PLFA 18:1ω8c, typical of *Methylosinus trichosporium*, increased to 16 to 28% of the total fatty acids (273, 274). It was suggested that type I methanotrophs dominate in environments that allow the most rapid growth of methane-utilizing bacteria while type II methanotrophs that tend to survive better are more abundant in environments where growth rates are restricted periodically by nutrient deprivation (394).

Roslev and King (324) reported that the type II methylotroph *Methylosinus trichosporium* OB3b survived up to 6 weeks of carbon deprivation under anoxic conditions and began to oxidize methane within hours after the addition of methane and oxygen. Another type II methanotroph also retained 36 to 118% of its methane oxidation capacity after 5 days of carbon deprivation, provided that oxygen was not present. Oxic conditions caused a loss of cell protein, morphological changes, and a loss of viability. Interestingly, these authors also observed that one isolate from peat, strain WP 12, was capable of limited anaerobic glucose metabolism in the absence of exogenous electron acceptors and survived for 100 days in the presence of glucose and yeast extract if the culture was protected from oxygen. These observations may explain how some methanotrophs can survive periods of methane deprivation in soils and rapidly oxidize methane when it is provided. Bender and Conrad (37) observed significant aerobic methane oxidation potentials in soils that had been anoxic for approximately 90 years. Most methanotrophic bacteria produce resting stages, and the exospores and cysts of some methanotrophs appear to be well adapted for survival in dry and nutrient-depleted environments (50, 209, 210, 324, 418).

Freshwaters. In methanogenic sediments overlain with oxic water, methanotrophic bacteria occur at sediment surfaces. The same applies to stratified eutrophic freshwater lakes, where the methane-oxidizing bacteria occur within the metalimnia between the anoxic and methane-saturated hypolimnia and the aerobic epilimnia (151, 158, 166, 297, 327–329). The highest populations of methanotrophic bacteria determined by plate counts (6×10^4 to 4.0×10^6) coincided with the highest rates of methane oxidation at the bottom of the chemocline in two lakes in Russia (188, 332). In rice paddies and vegetated wetlands, methanotrophic bacteria are abundant near root surfaces, where oxygen transported through the plants and methane produced in the sediments are both accessible (36, 37, 210, 318). Small numbers of methanotrophs were detected in flowing waters (400 to 800/ml) (332).

The dominant methanotrophs in the metalimnia of eutrophic lakes are members of the genus *Methylomonas*. Far smaller numbers of the type II methanotrophs, *Methylosinus trichosporium*, *Methylosinus sporium*, and *Methylocystis parvus*, were found in an oligotrophic lake (Lake Svente). Saralov et al. (332) studied nitrogen fixation under microaerobic conditions in two acidic (pH 4.4 to 5.4), polyhumic lakes, where they detected only the type II methanotrophs *M. trichosporium* and *M. sporium*. Only type II and type X methanotrophs fix nitrogen (48, 151, 152, 415, 416), and it is expected that these methanotrophs will dominate when the concentrations of combined nitrogen compounds are growth limiting (145). Under these conditions, the oxidation of methane and growth of methane-oxidizing bacteria are believed to be sensitive to dissolved oxygen levels above 20% of that found in waters in equilibrium with the atmosphere (151, 327, 328); for this reason, methane oxidation is confined to a narrow zone near the bottom of the metalimnia of some lakes, where the dissolved oxygen levels are below 1.0 mg liter^{-1}. Heyer and Suckow (174) also found that acidic lakes (pH 3.8 to 5.3), in which the high rates of methane oxidation (90 to 430 μM m^2 h^{-1}) occurred in peat sediments depleted in combined nitrogen, yielded only type II methanotrophs in enrichment cultures.

In eutrophic lakes that have high levels of nitrate and ammonia, like Lake Mendota, Madison, Wis., and Lake Minnetonka, Minn., only type I methanotrophs were detected in the metalimnion during summer stratification as evidenced by the hybridization of type I but not type II phylogenetic signature probes to total RNA extracted from metalimnion biomass samples (51a, 153). The amount of hybridization of the type I probes per microgram of RNA extracted from the water sample biomass was largest in samples taken from the metalimnion, where methane oxidation rates were the highest (153). Extracts prepared from the biomass collected from the metalimnion of Lake Minnetonka were assayed for key enzymes of the RuMP and serine pathways for formaldehyde fixation and were shown to contain hexulose-6-phosphate synthase (0.2 to 0.6 μM hexulose-6-phosphate formed min^{-1} mg of protein^{-1}). The extracts did not have detectable amounts of hydroxypyruvate reductase (153). These data were taken as further support for the dominance of type I methanotrophs in the metalimnion of this lake. The population of type I methanotrophs was estimated by determining the amount of the type I phylogenetic signature probe that bound to the RNAs extracted from the bacteria in the water samples compared with the amount of the eubacterial consensus phylogenetic signature probe that was bound to the same RNAs. The RNAs extracted from different depths within the epilimnion all hybridized with the eubacterial probe, but hybridization of the type I methanotroph signature probe was confined to RNAs extracted from metalimnion samples. The methanotrophs were estimated to make up approximately 1% of the eubacterial population within the metalimnion.

In oligotrophic lakes like Lake Steckin, Germany, where the entire water column is aerobic, methane oxidation and methanotrophs are confined to the sediment surfaces. Sediment pore water samples from Green Bay, Lake Michigan, rapidly oxidized methane (13 to 37 mM^{-1} h^{-1}), suggesting that a large population of methanotrophs was present in the sediments. The copper concentrations of the sediments were relatively low

(15 to 38 nM) in pore water and may limit the methane-oxidizing potential (58).

McDonald et al. (254) used PCR to amplify genes encoding sMMO components and the large subunit of MDH to detect methanotrophs in peat samples, freshwater pond sediments, and marine sediments. They found that the *mmoX* gene sequences (which encode the MMO hydroxylase alpha-subunit) in the PCR products recovered were conserved while predicted *mmoC* gene sequences (which encode the reductase component of MMO) were more variable. The *mmoX* sequences which were PCR amplified from peat DNA were compared with *mmoX* sequences from pure cultures at the predicted amino acid level. The 23 sequences derived from peat samples compared with the *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b hydroxylase alpha-subunits were 11.5 to 92.9% homologous, and similarities ranged from 30.8 to 94.6%, indicating that a large variety of methanotrophs were present in peat samples.

Aquifers. Groundwater collected from 13 monitoring wells on the U.S. Department of Energy Savannah River Site near Aiken, S.C., were enriched with methane, and 25 methanotrophic bacteria were isolated (46). PLFA profiles of all the isolates indicated that they all contained relatively high levels of 18:1ω8c fatty acids. Subsequent phenotypic testing indicated that most isolates were strains of *Methylosinus*, and one was identified as a strain belonging to the genus *Methylocystis*. Most exhibited sMMO activity as evidenced by the naphthalene oxidation assay, and a *mmoB* gene probe hybridized to DNA extracted from most isolates. Injection of methane and air into the subsurface groundwater resulted in enrichment of methanotrophic populations and methanotrophs that contained DNA sequences complementary to the *mmoB* gene (194, 301).

Oceans. Although methane oxidation rates and methane concentrations measured in the open ocean are very low, methanotrophic bacteria have been isolated from these environments (172, 174, 184, 229, 347). Marine strains encompass a narrow range of phylogenetic and phenotypic strain diversity among the isolates (180). One reason for the lack of diversity of cultured marine methanotrophs may be the inadequacy of the culture techniques used for their isolation. Marine methanotrophs are difficult to isolate compared with their terrestrial and freshwater counterparts (229, 231, 346, 347). *Methylomonas pelagica*, a typical type I methanotroph except for its salt requirement for growth and sensitivity to sunlight, was isolated from the Sargasso Sea (347). Two methanotrophs isolated by Lees et al. (229) also required salt and were otherwise typical obligate type I methanotrophs, except that they would not grow on solid media. Pure cultures were isolated after enrichment in mineral salts media supplemented with vitamins followed by dilution into "sloppy agar" plates containing 0.4% (wt/vol) Noble agar. The marine strains isolated by Lidstrom (231) contained both the RuMP and serine pathways for formaldehyde fixation. Only type I methanotrophs, which normally require more copper for growth, have been isolated from marine environments, yet Sunda and Gillespie (375) and Bruland et al. (54) have found that the ambient copper levels in estuarine seawater are on the order of 10^{-9} to 10^{-11} M or less. Copper is complexed to organic compounds in many environments, which limits its availability (375).

Gal'chenko et al. (140) detected 2.3×10^5 to 1.3×10^6 methanotrophic bacteria in sediments from the Black Sea. In the sediments, 65 to 70% of the methanotrophs detected by immunofluorescence microscopy were type I methanotrophs of the genera *Methylobacter* and *Methylomonas*. In water just above the sediments, *Methylobacter* species and type II methanotrophs of the genus *Methylocystis* were most abundant.

Bowman et al. (48) have sequenced the 16S rRNAs of several methanotrophs including *Methylomonas pelagica*, which they renamed *Methylomicrobium pelagicum* because of its relationship to other members of this new genus by phenotypic and phylogenetic comparisons (48, 49). Antibodies prepared against killed cells of *Methylomicrobium pelagicum* cross-reacted with *Methylomonas methanica* (346).

The endosymbiont of mytilid mussels growing near hydrocarbon seeps in the Louisiana Slope of the Gulf of Mexico formed a distinct branch within the family *Methylococcaceae*. The depth of the branch in the phylogenetic tree indicated that this endosymbiont represented a new genus of type I methanotrophs (49, 65, 114).

Because of the difficulties in isolating diverse methanotrophs from marine environments, Murrell's group has used 16S rRNA probes to observe changes in enrichments in marine waters enriched with methane and low concentrations of essential nutrients (180). Holmes et al. (180) enriched for methanotrophs in seawater samples by addition of methane and essential nutrients. The enrichments contained pink pellicles characteristic of *Methylomonas* spp. A 16S ribosomal DNA (rDNA) genomic library was constructed from the bacterial populations by cloning of PCR products amplified with primers for 16S rRNA genes. Previously described phylogenetic signature probes failed to give hybridization signals with RNA samples extracted from these enrichments. Sequencing of the cloned 16S rDNAs indicated that novel methanotrophs related to *Methylomonas* spp. were present in the enrichment cultures. Specific probes were designed to hybridize with unique sequences in the 16S rRNA of this bacterium. Sequences complementary to the unique sequences in the 16S rRNA of this bacterium were found in subcultures as well as in the original enrichment culture. The probes, when labeled with the fluorescent dye rhodamine, hybridized to a single morphotype in the enrichments. These cells made up about 45% of the cells in the enrichment culture. These experiments demonstrated that phylogenetic signature probes derived from laboratory strains cannot always be relied on to detect all the microbes within a physiological group that are present in an environmental sample and that cloning and sequencing of rDNAs is a good alternative for characterization of the populations present in some habitats and enrichments.

Attempts to enrich for methanotrophic bacteria from microbial mats from the hypersaline Solar Lake, Sinai, Israel (83), or to measure methane oxidation at 9% salinity failed, although oxygen and methane were both present. Methane production rates in the mats were 0.4 to 2.2 nmol h^{-1} cm^{-2} (82) and were unaffected by the presence or absence of air or when inhibitors of methane oxidation were added to the mat samples. Slobodkin and Zavarzin (354) also failed to detect methane oxidation in hypersaline cyanobacterial mats of Lake Sivash.

Factors Influencing Competition between Type I and Type II Methanotrophs

Amaral et al. (16, 17) have used semisolid agarose diffusion columns with opposing gradients of methane and air for the isolation of methanotrophic bacteria from a variety of natural sources and to study the associations between methanotrophic bacteria and denitrifying bacteria. The methane and oxygen gradients within the columns closely mimicked natural environments. It was reasoned that these diffusion columns might allow the isolation of more or different methanotrophs than would other enrichment cultures and that they might provide information of value for studying the ecology of methane-utilizing bacteria. Bands of growth associated with methane

oxidation activity were observed near the top of the columns, where the concentration of methane was low and the concentration of oxygen was high. DNA was extracted from the bands and was hybridized with phylogenetic signature probes for type I and type II methanotrophs. The results indicated a preponderance of type I methanotrophs in bands near the top of the columns. In some columns, bands developed near the bottom of the columns because of contamination of the methane with oxygen or leakage of oxygen into the columns at the bottom. The dissolved oxygen concentrations were very low at the bottom of the columns, and methane concentrations were high. Hybridization experiments indicated that these bottom bands contained type II methanotrophs. The authors proposed that type I methanotrophs outcompeted type II methanotrophs at low methane concentrations whereas the growth of type II methanotrophs was favored under low-oxygen, high-methane conditions. Their hypotheses have been supported by observations that a type I methanotroph, *Methylomonas albus* BG8, outcompeted a type II methanotroph, *Methylosinus trichosporium* OB3b, in continuous cultures under methane-limiting conditions (145). Growth of the type II methanotroph was favored under nitrogen- or copper-limiting conditions. Methanotrophs found at the metalimnia of two eutrophic freshwater lakes which contained relatively high levels of combined nitrogen were shown by 16S rRNA hybridization analysis with phylogenetic signature probes to be type I methanotrophs (153). The methane oxidation activity was detected at the top of the methane gradient of the chemocline, and it is assumed that the type I methanotrophs grow more rapidly than type II methanotrophs under these conditions. In Cleveland Harbor, *Methylomonas methanica*, a type I methanotroph, was most abundant in the water column, while *Methylosinus trichosporium*, a type II methanotroph, was found only at the surface of methanogenic sediments (319). Methanotrophs associated with the rhizosphere of aquatic plants, where methane concentrations are high, are type II methanotrophs (211). When soils were exposed to methane air atmospheres, type II methanotrophs dominated the enrichments as shown by PLFA analysis (273) and by hybridization of 16S rRNA extracted from soils with phylogenetic signature probes (55). These observations support the hypothesis that the concentrations of methane, oxygen, and combined nitrogen are the primary determinants of the type of methanotrophs present in an environment (16, 17), although soils rich in organic matter which combines with copper may favor the growth of type II methanotrophs (145).

Associations of Methanotrophs with Other Bacteria, Invertebrates, and Wetland Plants

It has long been known that bacteria capable of growth on methanol and organic compounds excreted by methanotrophs copurify with them during attempts to obtain pure cultures (151). Hyphomicrobia are common consorts in enrichment cultures. Methanol produced during the oxidation of methane is toxic to most obligate methanotrophs, and associated hyphomicrobia utilize the methanol (151, 158, 160, 381). Other consorts utilize organic materials excreted by methanotrophs and often supply growth factors, including vitamins, required by methane-utilizing bacteria (222, 270).

Amaral and Knowles (17) have demonstrated that denitrification and N_2O production occur in anaerobic zones of their agarose columns, with countergradients of methane and oxygen (see above). The methane-dependent production of N_2O has been attributed to the use of organic matter produced as exometabolites or lysis products by methanotrophs and the utilization of oxygen by the methanotrophs to produce anoxic zones required for denitrification (16, 33, 257). Thus, methane-oxidizing bacteria may indirectly contribute to the production of the radiatively active trace gas N_2O to the atmosphere. Similar associations between methanotrophs and sulfate reducers in marine sediments and flooded oil reservoirs have been proposed (140).

Endosymbiotic methanotrophic and chemoautotrophic bacteria are the basis for invertebrate communities surrounding deep-sea hydrothermal vents and cold gas seeps in the Atlantic and Pacific Oceans (64–66, 70, 86, 128–130, 323). The symbioses provide the symbionts simultaneous access to substrates (methane and reduced sulfur compounds) and an oxidizing environment, while the eukaryotic partners derive organic compounds for growth. Symbiotic associations with methanotrophic bacteria have been described for three species of invertebrates, including two different and unnamed species of deep-sea mytilid mussels (family Mytilidae) found in the Gulf of Mexico near cold gas seeps and pogonophoran tubeworms (*Siboglinum poseidoni*) from the Central Skagerrak (64, 70, 128–130, 333). The invertebrates occur in sediments where methane (up to 10 mM) and oxygen are both present at areas of cold hydrocarbon seeps and hydrothermal vents (65). In the mussels, the methanotrophs are present as intracellular endosymbionts in the gill tissue. The endosymbionts were first observed within the mytilid gills and pogonophoran trophosome tissues by transmission electron microscopy. The symbionts appeared to be gram-negative cells with stacked arrays of intracytoplasmic membranes similar to those found in type I and type X methanotrophs and some nitrifying bacteria (65). The oxidation of [^{14}C]methane to $^{14}CO_2$ and the incorporation of methane carbon into acid stable compounds by tissue samples have been demonstrated in tissues of mussels and tubeworms. Net methane consumption was detected in fresh gill tissues of both mytilid and pogonophoran trophosome tissues, as was methanol dehydrogenase and hexulose-phosphate synthase, an enzyme which is present exclusively in type I and type X methanotrophs (64–66). Stable-isotope composition of the mussel tissues also indicated that methane was a major source of carbon in the invertebrates (65, 358).

The symbionts of invertebrates have not been cultivated on artificial media. Distel et al. (114) have analyzed the phylogenetic relationship of the endosymbionts in the tissues of mytilids from two sites by comparison of their 16S rRNA sequences with the sequences of 16S rRNAs of other eubacteria including several methanotrophs. Their analysis and that of Bowman et al. (48) indicate that the sequences of endosymbionts from the Florida and Louisiana mytilids form a separate cluster most closely affiliated with other type I methanotrophs. Bowman et al. have indicated that the phylogenetic data support the classification of these symbionts as new genera within the family *Methylococcaceae.*

Other hydrothermal vent mytilids and pogonophorans possess sulfide-oxidizing chemoautotrophs as intracellular symbionts (128, 129), and two reports (64, 129) have described the cooccurrence of methanotrophic and chemoautotrophic sulfur-oxidizing bacterial symbionts in a deep-sea mussel. Two types of bacteria were found within the same cells of gill epithelial tissues of the Mid-Atlantic Ridge mussels (65).

Hovland and Judd (183) have described cold-water coral reefs in the North Sea off Norway. These corals exist over gas seeps, are devoid of algae that are normally found in tropical and shallow water corals, and develop independently of sunlight. It has been proposed that bacteria grow in the sediments and seawater around the seeps and are a source of nutrition for plankton. Methane and other minerals have been proposed as the major source of nutrients for filter feeders and shell-form-

ing organisms that accumulate at the seepage sites. Their skeletal remains and sediment grains are cemented together to form reefs. These reefs form at seepage sites throughout the oceans (183).

King (211) has shown that sediment-free roots of many different aquatic macrophytes associated with wetlands oxidize methane at rates of 1 to 10 mmol g (dry weight)$^{-1}$ h^{-1}. These rates correspond to 2×10^8 to 2×10^9 methanotrophs g (dry weight) of root tissue^{-1}. Phylogenetic signature probes for type II methanotrophs consistently hybridized to the RNAs extracted from plant roots to a greater extent than did probes specific for type I methanotrophs. The relative amounts of the phylogenetic probe specific for type II methanotrophs that hybridized with RNA from roots of the aquatic grass *Calamogrostis canadensis* were correlated with the rates of methane oxidation by root tissues. It was suggested that the rhizoplane and the interiors of roots of aquatic plants that are conduits for methane from methanogenic sediments to the atmosphere and for atmospheric oxygen to the sediments are both locations of substantial populations of methanotrophic bacteria.

Although we have not detected methane oxidation by bacteria attached to rhizomes or pads of water lilies, we did observe significant rates of oxidation by floating vegetation, particularly *Lemna minor* (little duckweed), in Minnesota wetlands (153). This plant, which grows in abundance on the surfaces of freshwater ponds, appears to harbor relatively large populations of methanotrophs that oxidize methane which would otherwise escape to the atmosphere. The plants provide oxygen, which is limiting in these anoxic shallow wetlands.

Anaerobic Methane Oxidation

An anaerobic methane cycle that operates in marine sediments has been estimated to consume 100 to 200 Tg of methane per year (7, 312–315). Several studies have confirmed that methane is also consumed in other anaerobic environments, including anoxic marine water, sediments of soda lakes, and freshwater sediments (7–9, 189, 190, 219, 293, 312–316). In vertical profiles of marine sediments, methane oxidation and sulfate reduction occur coincidentally (8, 9, 189, 190, 313, 315).

Mono Lake, Calif., is a permanently stratified meromictic lake. It is hypersaline, alkaline (pH 9.8), and rich in carbonate (0.5 M) and sulfate (110 mM), and it has been stratified year round since 1983 because of an influx of freshwater, which causes a decreased salinity at the surface water. Methane is produced in the sediments and diffuses upward (291). Most of the methane oxidation occurs in the anaerobic bottom waters and sediments. In another alkaline saline lake, Big Soda Lake, Nev., Iversen et al. (190) observed oxidation rates of 49–85 nM liter^{-1} day^{-1} in anoxic bottom waters whereas the rates of oxidation in the oxic layers were much lower (1.3 nM liter^{-1} day^{-1}). The zones of methane oxidation and sulfate reduction coincided in the water column (190).

Anaerobic methane oxidation has frequently been determined by measuring the conversion of [^{14}C]methane to $^{14}CO_2$ which is trapped in alkaline solutions (8, 9, 152, 293, 313, 315, 432, 433). The methane has usually been prepared by converting labeled CO_2 and hydrogen to labeled methane with the methanogen *Methanobacterium thermoautotrophicum* (104). In nearly all cases, net methane consumption was not demonstrated when pure cultures were used. Harder (159) has recently shown that radioactive methane prepared in this manner contains 0.2 to 1.0% carbon monoxide because some methanogens employ this compound as an intermediate in the biosynthesis of acetate (85, 426). Therefore, some of the presumed methane oxidation could, in part or in total, be the result of the oxidation of labeled carbon monoxide to CO_2. Carbon monoxide is known to be oxidized by several anaerobes (426). Harder (159) eliminated the carbon monoxide from methane and retested the ability of several pure cultures of anaerobes to oxidize methane. Only cultures of methanogenic bacteria converted the labeled methane to carbon dioxide. Known sulfate-reducing bacteria failed to do so.

Panganiban et al. (293) enriched for anaerobic methane-utilizing bacteria by using a mineral salts medium containing acetate or lactate as the supplemental carbon source. In the cultures isolated from lake sediments, growth was dependent on the simultaneous presence of methane and sulfate and required acetate or lactate as an additional carbon source. Methane oxidation was dependent on the presence of sulfate, and sulfate reduction required methane as a reductant (151). Methane was required for the reduction of sulfate to sulfide, for the growth of liquid cultures, and for the development of colonies on agar. Sulfate was required for the oxidation of [^{14}C]methane to $^{14}CO_2$, and the oxidation of methane was inhibited by traces of oxygen. Methane carbon was not incorporated into cell material. Hoehler et al. (177) have suggested that a consortium of methanogens and sulfate-reducing bacteria is responsible for anaerobic methane oxidation in anoxic sediments from Cape Lookout Bight, N.C.

Pure cultures capable of anaerobic methane oxidation have not been characterized, and the biochemistry of the process remains to be described.

BIODEGRADATION OF TOXIC CHEMICALS BY METHANOTROPHIC BACTERIA

Methane and methanol have been important feedstocks for the chemical industry for many years. Interest in the production of single-cell protein by methylotrophs, the isolation and characterization of a large number of new methane-utilizing microbes, and studies of their physiology in the 1960s and 1970s stimulated the interest of applied microbiologists and resulted in funding of research that has helped sustain an interest in these microbes to the present (101, 106, 150, 152, 175, 223, 308, 417). However, methanol has largely replaced methane as a potential feedstock for industrial fermentations involving methylotrophic bacteria. Methanol is abundant, stably priced, easily produced from methane, relatively safe to use, and easily stored and transported. In addition, a large number and diversity of microbes grow on methanol as a source of carbon and energy (20, 21, 112, 150, 223, 232, 236). The unique abilities of the MMOs of methanotrophic bacteria to catalyze some reactions of environmental and perhaps commercial importance have sustained the interest of applied microbiologists in the methanotrophic bacteria. At this time, there are no profitable processes employed by industries to produce chemicals like propylene epoxide that have appeared very promising (223, 236). However, the discovery that methanotrophic bacteria can degrade low-molecular-weight halogenated hydrocarbons that are ubiquitous and toxic pollutants has resulted in research activities that may lead to their greatest potential for commercial application at present (10, 56, 123, 133, 167, 193, 253, 255, 283, 284, 386).

Chlorinated Hydrocarbons as Pollutants

Synthetic chlorinated hydrocarbons have been used as solvents because of their stabilities, solvent properties, and low potential for flammability and explosion. As a result, they have been used in degreasing metals and electronic components in semiconductor manufacturing, in dry cleaning, as propellants,

as fumigants for the control of pests and fungi, as extractants of caffeine from coffee, in general anesthetics, and in manufacture of plastics (123, 154, 193). Some chloromethanes are products of reactions that occur during water chlorination (395). As a result of the widespread use and careless handling, storage, and disposal of solvents, coupled with their chemical stabilities, they have become the most frequently detected groundwater contaminants in the United States and other countries (123, 143, 154, 240, 300, 407). The most ubiquitous of these chemical pollutants are trichloroethylene (TCE), 1,2-dichloroethane, and chloroform (407). TCE is the most frequently encountered in groundwaters, and it has a half-life of 300 days in one aquifer (143). It is a suspected carcinogen in humans (260, 393) and is directly toxic or carcinogenic in yeasts and in in vitro assays (352, 395). Tetrachloroethylene and dichloroethylene, which are also frequently encountered in groundwaters, are, like TCE, converted to vinyl chloride by bacteria under anaerobic conditions that exist in landfill sites and some groundwaters (395, 396). Vinyl chloride is a known carcinogen in mammals (187, 246).

Degradation of Chlorinated Hydrocarbons by Bacteria Containing Nonspecific Oxygenases

Bacteria that will utilize trichloroethylene or chloroform as a sole carbon and energy source have not been discovered (152). However, several bacteria that contain nonspecific oxygenases, such as MMOs, cause the mineralization of these compounds through processes referred to as cometabolism (cooxidation) or fortuitous metabolism (97, 101, 123, 154, 397).

Wilson and Wilson (420) first demonstrated that microbes in unsaturated soil columns which had been exposed to natural gas converted TCE to carbon dioxide and chloride under aerobic conditions. Columns that were not exposed to natural gas did not degrade TCE. These authors suggested that methanotrophic bacteria were responsible for its destruction. Several subsequent studies have shown that methanotrophs, other bacteria, and tissues that contained nonspecific monooxygenases and diooxygenases are capable of transforming a variety of halogenated aliphatic compounds (45, 68, 123, 131, 133, 154, 240, 260, 382, 395, 397, 398, 419, 421). Fogel et al. (131), and Janssen et al. (193) have shown that mixed cultures enriched by growth on methane degraded several chlorinated derivatives of methane, ethane, and ethylene. Vinyl chloride was degraded more rapidly than dichloroethylene and TCE, whereas dichloromethane was oxidized faster than chloroform (45, 131, 253). The rates and extent of aerobic degradation of chlorinated compounds for any series (ethanes, ethylenes, and haloforms) are inversely related to the chlorine/carbon ratios (68). Highly chlorinated hydrocarbons including tetrachloroethane, tetrachloroethylene, and carbon tetrachloride were not degraded by these consortia of aerobic bacteria (118, 262). These highly chlorinated species were reductively dehalogenated by nonenzymatic reactions involving coenzymes and transition metal complexes in anaerobic bacteria, yielding products that could be attacked by aerobic species containing nonspecific oxygenases (118, 220, 138). Sequential conversions of tetrachloroethylene to TCE, vinyl chloride, and ethylene under anaerobic conditions has been demonstrated (138, 262, 374). The rates of reductive dechlorination by methanogenic bacteria decreased in microcosm studies as the degree of chlorination decreased (138, 262, 374).

Degradation of Trichloroethylene by Methanotrophic Bacteria and Consortia

Little et al. (240) reported the oxidation of TCE by a pure culture of a methane-utilizing bacterium and suggested that it was oxidized to TCE epoxide by a reaction similar to the known conversion of propylene to propylene expoxide by MMO (101, 176). The rates of this conversion were low (<0.1 nmol min^{-1} mg of cell dry $weight^{-1}$) (240). Oldenhuis et al. (283, 284) and Tsien et al. (386, 387) found that rates of TCE degradation by cells of *Methylosinus trichosporium* OB3b were at least 2 orders of magnitude higher (>150 nmol min^{-1} mg of cell $protein^{-1}$) than by cells of other bacteria which contained nonspecific monooxygenases, provided that the cells were grown with limiting copper. The rates of TCE degradation correlated with levels of sMMO, and cells grown with higher levels of copper did not contain sMMO or oxidize TCE (154, 283, 284, 386). High rates of TCE oxidation required formate as a reductant to supply additional reducing power for sMMO (154, 283, 284, 386). Fox et al. (133) measured the oxidation of TCE by using purified components of sMMO and observed that all components and NADH + H^+ were necessary to obtain very high rates of oxidation. The rate of TCE oxidation observed was 680 nmol min^{-1} mg of $protein^{-1}$ with a K_m of 35 μM. Both whole cells of *M. trichosporium* and the purified sMMO also oxidized dichloroethylene, dichloroethanes, and vinyl chloride (133, 386).

We (56, 154, 386, 387) examined several methanotrophic bacteria for their abilities to oxidize TCE and observed that four species of type II methanotrophs classified in the genus *Methylosinus* and the type X methanotroph *Methylococcus capsulatus* (Bath) were able to degrade this compound. Two type II bacteria classified within the genus *Methylocystis* and type I methanotrophs failed to oxidize TCE. The ability to oxidize TCE was found only in cells which were shown to possess sMMO as evidenced by the presence of proteins that cross-reacted with antibodies prepared against the hydroxylase component of sMMO, their abilities to oxidize naphthalene to naphthols, and the presence of DNA that hybridized to the *mmoB* gene probe (154, 387). Recently, Koh et al. (218) showed that a type I methanotroph which also possesses sMMO when grown with limiting copper is capable of rapid TCE oxidation. Much lower rates of TCE oxidation have been reported for bacteria that possess pMMO (113).

Methylosinus trichosporium OB3b was shown to oxidize TCE much more rapidly than did cells of nitrifying bacteria that possess AMO (25), cells of *Pseudomonas cepacia* that possess toluene 2-monooxygenase (132), *Pseudomonas mendocina* containing toluene 4-monooxygenase (421), cells of *Pseudomonas putida* F1 containing toluene dioxygenase, and cells of *Mycobacterium* sp. with propane monooxygenase (398).

The products of TCE oxidation by sMMO were formate, CO, glyoxylate, dichloroacetic acid, and chloral (123, 133). The first reaction product is trichloroethylene epoxide, which is rapidly transformed to glyoxylic acid with the removal of chloride (123, 169, 255). Glyoxylic acid is chemically or biologically oxidized to carbon dioxide. The oxidation of TCE results in destruction of purified sMMO. Complete inactivation occurred after 200 molecules of TCE were oxidized per molecule of hydroxylase component, and carbon from [^{14}C]TCE was shown to be covalently attached to all of the three hydroxylase subunits (123, 133). The inactivation of MMO in intact cells during the course of TCE degradation has also been demonstrated (11, 12, 283), and TCE transformation products were bound nonspecifically to cell proteins (283). The toxicity of the products of TCE metabolism was reduced somewhat by the addi-

tion of formate to provide additional reducing equivalents (283, 359).

TCE is completely degraded by mixed cultures of bacteria that grow with methane as the sole carbon and energy source (53, 68, 390). The transformation capacities (mass of TCE degraded per unit mass of cells) of mixed cultures containing methanotrophic bacteria (0.5 mg of TCE mg of cells^{-1}) are higher than for mixed cultures of bacteria grown with toluene, propane, or phenol (68). It is believed that consorts in the mixed cultures assist in the removal of the partial degradation products of TCE metabolism. In one study, the addition of *Xanthobacter autotrophicus* to *Methylocystis* sp. strain M to reconstruct a mixed culture resulted in more complete removal of the water-soluble products of TCE metabolism (390).

Alvarez-Cohen et al. (10, 12, 13) have studied the degradation of TCE by a mixed culture of bacteria enriched with methane and oxygen from aquifer material from Moffett Field Naval Air Station, Mountain View, Calif. When the culture was grown in a bioreactor under methane and nitrogen limitation, it rapidly oxidized TCE (0.6 to 1.1 mg of TCE mg of cells^{-1} day^{-1}) and chloroform (0.4 mg of chloroform mg of cells^{-1} day^{-1}). The culture was stable for several years, although the media and reactors were not sterilized. The culture consisted of a crescent-shaped bacterium, a motile rod-shaped bacterium, and prosthecate bacteria resembling *Hyphomicrobium* species (13). A pure culture of a methanotrophic bacterium was isolated from the enriched culture. The crescent-shaped bacteria in the pure culture and the mixed culture were morphologically indistinguishable by microscopic examination; cells of both cultures hybridized with fluorescent phylogenetic signature probes for type II methanotrophs (13); and *Ase*I restriction fragments produced by digestion of DNA extracted from cells in the pure culture and the bioreactor that hybridized to the *mmoB* gene probe were identical in size and were different in size from *Ase*I fragments resulting from digestion of DNAs from other methanotrophic bacterial pure cultures (13). Both the pure culture and the cells in the bioreactor produced sMMO as shown using the naphthalene oxidation assay. Therefore, it was concluded that the cells in the pure culture were identical to the methanotrophs in the bioreactor. The sequence of the 16S rRNA from the pure culture indicated that the methanotrophic bacterium was most closely related to the type II methanotroph, *Methylosinus sporium.*

Applied Aspects of Methanotrophic Trichloroethylene Degradation

Several different bioreactor configurations for the degradation of TCE and other halogenated hydrocarbons by *Methylosinus trichosporium* have been described (10, 11, 13, 53, 167, 253, 255, 303, 349, 359, 369). Excellent reports of conditions that have been used to enhance bioremediation in situ and in bioreactors have been published (167). Competition between methane and chlorinated solvents for the active site of sMMO, inactivation of cells by reactive metabolites of halogenated hydrocarbons, and reducing-equivalent supply for the sMMO-catalyzed reaction all cause complications in attempts to use methanotrophs for bioremediation of these chemicals in some reactor configurations (11, 53, 167). Methane must be supplied to sustain the biomass, and formate is usually added as an additional source of reducing power (10–12, 167, 283, 284, 359, 386). Sequencing bioreactors which cycled between growth and degradation modes of operation have been described for the degradation of chloroform and TCE (10, 359). In one configuration, the cells were grown in a packed-bed reactor with methane and oxygen, and chloroform (100 μg liter^{-1}), and formate (8 mg liter^{-1}) were then supplied without methane. This configuration performed better than a packed-bed, continuous-flow reactor (359). Degradation rates in the degradation stage were sustained for 2 weeks without loss of activity, but they decreased after this time. However, the specific rates of chloroform degradation were 2 orders of magnitude lower than those observed with suspended cultures.

The feasibility of in situ bioremediation by stimulation of the growth of indigenous populations of methanotrophs has been investigated by Semprini and McCarty (349) at the Moffett Naval Air Station and by Hazen (167) at the U.S. Department of Energy Savannah River Site. Semprini and McCarty (349) reported that biodegradation of TCE occurred within the Moffett Field aquifer after it had been supplied with methane and oxygen to stimulate indigenous methane-oxidizing bacteria. Methane and air mixtures were also injected into the Savannah River Site by horizontal well injection technology with the intention of stimulating methanotrophic TCE degradation (167, 301). The initial results indicated that a substantial increase in type II methanotrophic bacterial populations occurred in some areas of the aquifer, and an increase in the frequency of bacteria that contained DNA with sequences complementary to the *mmoB* gene at different sites was observed (46).

We have observed that several floating aquatic plants at the Minnesota Landscape Arboretum oxidized methane and TCE. *Lemna minor* (little duckweed) oxidized these compounds at specific rates that were 1 order of magnitude higher than the specific oxidation rates observed with other floating vegetation (153). This plant forms a confluent layer on the surface of wetland ponds during the summer. TCE oxidation by plant material (0.4 nmol g^{-1} day^{-1}) was detected consistently during the summer months (153). The methanotrophic bacteria associated with these plants contained sMMO as evidenced by the ability of the unwashed plants and bacteria washed from plant surfaces to oxidize naphthalene to naphthols, hybridization of DNA extracted from the unwashed plants to the *mmoB* gene probe, and hybridization of RNA extracted from the unwashed plants and bacteria washed from them to phylogenetic signature probes specific for methanotrophic bacteria (153).

Methanotrophic bacteria have also been shown to degrade methyl bromide and methyl fluoride (275). The rates of degradation varied inversely with the methane supply. Methyl bromide is converted to very toxic products, and this compound has been employed as a suicide substrate for the isolation of mutants that do not synthesize MMO (275).

Mixed cultures of methanotrophs isolated from an uncontaminated groundwater aquifer transformed 2- and 4-chlorobiphenyl and 4-hydroxy-2-chlorobiphenyl to hydroxychlorobiphenyls (3). It is believed that some methanotrophs can initiate degradation of the less chlorinated biphenyls. In mixed cultures, the transformation products were further degraded by ring cleavage. These reactions were attributed to heterotrophic bacteria in the mixed cultures.

CONCLUSIONS AND PROSPECTS

The methanotrophs possess unique enzymatic activities for the oxidation of methane and assimilation of the resulting one-carbon units which are encoded by unique genes. Some of these genes, including those encoding the sMMO hydroxylase and MDH, can be used to monitor populations of methylotrophs in different environments by molecular techniques. The division of type I, II, and X methanotrophs has been justified phylogenetically. These divisions have been shown to have an ecological basis for type I and II; the two groups

appear to be adapted for survival under different nutrient-limiting conditions. The type I methanotrophs appear to be best adapted to grow at low methane concentrations. The growth of some type II methanotrophs is favored when methane levels are high, when combined nitrogen and oxygen levels are low, and when copper is substantially depleted in the growth media. It is interesting, and perhaps fortunate, that the conditions that exist in groundwaters appear to favor the growth of the type II methanotrophs and the synthesis of sMMO that is essential for the rapid degradation of TCE and some other low-molecular-weight halogenated hydrocarbons. The use of these bacteria for in situ bioremediation appears to be an attractive possibility.

We have seen that methanotrophs are ubiquitously distributed and play a significant role in the global methane budget and therefore in moderating the impact of methane on global warming. They oxidize most of the methane produced in anaerobic environments before it reaches the atmosphere, and in unsaturated soils they oxidize significant amounts of atmospheric methane. Increased rates of methane oxidation and decreases in methane flux may occur as northern latitudes dry during global warming. Therefore, organic carbon would be mineralized and the impact of methane on global warming would be reduced. However, it is extremely difficult to predict climatic changes and the nature of the habitats that will exist in the future given our current state of knowledge.

We have not exhausted the interesting possibilities for discovery of new microbes and new biochemical processes. The microbes responsible for the oxidation of atmospheric methane are not known and appear to contain methane oxidation systems with different kinetic properties from those that have been characterized. The availability of molecular techniques for examining these environments has allowed us to characterize in a limited way the microbes that exist in some habitats from which part or all of the methanotrophic communities have not been isolated.

The anaerobic methane-oxidizing organisms are of great importance, yet we know little of them. It is possible that acetogenesis from CO and methane followed by acetate oxidation by sulfate-reducing bacteria can account for these reactions. It is also possible that new biochemistry is involved in these processes.

Environmentally, methanotrophs can form the base of marine food webs that are independent of sunlight and can form symbiotic interactions with a variety of animals at these sites. It will be interesting to see if there are any nonmarine symbioses similar to those found near vents and cold gas seeps. Plant methanotroph associations may be very important in wetlands and other habitats. We know little of the value of the associations to the plants. No studies of axenic plant populations have been reported. Methanotrophs and methanotrophic consortia are also recognized as being among the most capable systems for the bioremediation of sites contaminated by chlorinated hydrocarbons.

A major question that confronts all of microbiology, no less than methanotrophs in particular, is the following. Are organisms currently in culture representative of those in the environment in diversity and behavior? The discovery of apparently new branches of type I methanotrophs in free-living and endosymbiotically associated marine systems argues that current cultures do not encompass existing diversity. Similarly, why have no marine type II methanotrophs been cultured, even though they are detectable by molecular methods? The marine environment of the deep ocean, with its low combined nitrogen levels and low copper levels, should provide a competitive advantage to type II methanotrophs as we understand the nutrition of the two groups.

Do the kinetics of methane oxidation in soils at low methane concentrations, which would not be predicted by the behavior of laboratory strains, indicate undiscovered strains or reveal previously unknown functionalities of known strains?

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