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Molecular Analysis of Deep-Sea Hydrothermal Vent Aerobic Methanotrophs by Targeting Genes of 16S rRNA and Particulate Methane Monooxygenase

Hosam Easa Elsaied, Toru Hayashi, and Takeshi Naganuma

School of Biosphere Sciences, Hiroshima University, Higashi-hiroshima 739–8528, Japan

Abstract: Molecular diversity of deep-sea hydrothermal vent aerobic methanotrophs was studied using both 16S ribosomalDNA and *pmoA* encoding the subunit A of particulate methane monooxygenase (pMOA). Hydrothermal vent plume and chimney samples were collected from back-arc vent at Mid-Okinawa Trough (MOT), Japan, and the Trans-Atlantic Geotraverse (TAG) site along Mid-Atlantic Ridge, respectively. The target genes were amplified by polymerase chain reaction from the bulk DNA using specific primers and cloned. Fifty clones from each clone library were directly sequenced. The 16S rDNA sequences were grouped into 3 operational taxonomic units (OTUs), 2 from MOT and 1 from TAG. Two OTUs (1 MOT and 1 TAG) were located within the branch of type I methanotrophic γ-Proteobacteria. Another MOT OTU formed a unique phylogenetic lineage related to type I methanotrophs. Direct sequencing of 50 clones each from the MOT and TAG samples yielded 17 and 4 operational *pmoA* units (OPUs), respectively. The phylogenetic tree based on the pMOA amino acid sequences deduced from OPUs formed diverse phylogenetic lineages within the branch of type I methanotrophs, except for the OPU MOT-*pmoA*-8 related to type X methanotrophs. The deduced pMOA topologies were similar to those of all known pMOA, which may suggest that the *pmoA* gene is conserved through evolution. Neither the 16S rDNA nor *pmoA* molecular analysis could detect type II methanotrophs, which suggests the absence of type II methanotrophs in the collected vent samples.

Key words: methane oxidation, phylogenetic, 16S rDNA, *pmoA*.

INTRODUCTION

Methanotrophs, gram-negative bacteria that utilize methane (CH_4) as their sole of carbon and energy, are classified into 2 major groups, aerobic methanotrophic bacteria and anaerobic methanotrophic archaea (Hinrichs et al., 1999). The aerobic methanotrophs are considered to be the largest biological sink for methane and other toxic compounds (DiSpirito et al., 1992). Taxonomically, aerobic methanotrophs are divided based on 16S ribosomal DNA analysis, cellular structures, and physiology of metabolism of methane into 3 types, I, II, and X (Hanson and Hanson, 1996). Both types I and X belong to γ-Proteobacteria, while type II is related to α-Proteobacteria (Galchenko and Andreev, 1984). Aerobic methanotrophic bacteria are known to possess the unique enzyme methane monooxygenase, which catalyzes the oxidation of methane to

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Corresponding author: Takeshi Naganuma; e-mail: takn@hiroshima-u.ac.jp

methanol (Anthony, 1982). The enzyme exists in soluble and membrane-bound particulate forms (Murrell, 1994; Semrau et al., 1995). The particulate form (pMMO) further consists of the subunits A, B, and C, which are encoded by 2 or 3 copies of a gene cluster bearing *pmoA*, *pmoB*, and *pmoC* (Stolyar et al., 1999; Gilbert et al., 2000).

Ecologically, deep-sea methanotrophic bacteria occur as free-living and symbiotic bacteria in a variety of gutless animals (e.g., Cavanaugh, 1993). Molecular studies of the deep-sea methanotrophs have focused mostly on only the phylogenetic gene 16S rDNA (Distel and Cavanaugh, 1994; Fujiwara et al., 2000); however, functional genes such as *pmoABC* have not been fully studied yet. Since the deduced pMMO subunit A (pMOA) harbors the amino acids corresponding to the catalytic site and shows conservation within all known aerobic methanotrophs, it may be used to detect aerobic methanotrophs in wide ranges of habitats (Dalton, 1991; Holmes et al., 1995a; McDonald and Murrell, 1997).

We studied the molecular diversity of the aerobic methanotroph populations in 2 contrastive (mid-oceanic and back-arc) vent microbial habitats by sequencing both 16S rDNA and *pmoA*. The dual gene analyses facilitate understanding the structures and functions of the aerobic methanotroph populations involved in the methane dynamics in the vent environments.

Materials and Methods

Deep-Sea Sampling

Two different deep-sea hydrothermal vent samples were studied. Hydrothermal vent plume and chimney fragments were collected from the Mid-Okinawa Trough (MOT), Japan, and the Trans-Atlantic Geotraverse (TAG) hydrothermal mound along the Mid-Atlantic Ridge, respectively. Details of the sample collection are described in Elsaied and Naganuma (2001).

DNA Sequence Analyses

Bulk genomic DNA was extracted from the samples according to Elsaied and Naganuma (2001). The PCR forward primers MG-64F (5′-GAA CGG TAA CAG GCC TTC GG-3′) targeting type I and X methanotrophs (γ-Proteobacteria) and MA-221F (5′-CGA AAG ATC GGC CCG CGT CC-3′) targeting type II methanotroph (α-Proteobacteria) (Bourne et al., 2000; Gulledge et al., 2001) were used to amplify partial 16S rDNA sequences of about 1438 bp and 1255 bp, respectively, with the universal eubacterial primer 1492R (Lane et al., 1985). The polymerase chain reaction (PCR) mixtures and conditions were performed according to Bourne et al., (2000).

The PCR primers A189 (5′-GGNGACTGGGACTTCTGG-3′) and A682 (5′-GAASGCNGAGAAGAASGC-3′) have the ability to amplify about 530 bp of the gene *pmoA* from all known methanotrophic bacteria (Holmes et al., 1995b; Semrau et al., 1995).

The PCR-amplicons were cloned using TOPO TA Cloning kit (Invitrogen). Fifty clones from each clone library having the target insert were analyzed by direct DNA sequencing.

The obtained DNA sequences were homology-searched on DDBJ using the program FASTA 3. The 16S rDNA sequences having 100% nucleotide identity were grouped as one single operational taxonomic unit (OTU). The *pmoA* nucleotide sequences were translated into deduced amino acids using the program Transq (European Bioinformatics Institute). The deduced amino acid sequences that showed 100% amino acid identity were grouped into a single operational *pmoA* unit (OPU).

The expected locations of transmembrane-spanning regions and topology of the deduced pMOA proteins were calculated using the TMHMM tool at the Swiss Institute of Bioinformatics, Expasy website (*http://www.expasy.ch/tools/#transmem*).

The phylogenetic trees were constructed by the neighbor-joining method (Saitou and Nei, 1987). The branching patterns of the constructed phylogenetic trees were confirmed by reconstruction of the phylogenies using maximum-parsimony and maximum-likelihood, contained within the PHYLIP package (Felsenstein, 1989).

All the recovered sequences in this study were deposited in DDBJ with accession numbers shown in Table 1.

Results and Discussion

Sampling Sites

Two contrasting vent sites were selected for sampling, one from a Pacific back-arc and the other from the Atlantic mid-oceanic system. The hydrothermal vent plume at MOT is rich in methane as abundant as 14.8 mM, which is 50 to 100 times higher than those at mid-ocean ridges

Table 1. Original Sample Sequence Taxonomic Units and Accession Numbers

Sampling site	Operational unit	Accession number	Number of clones
Mid-Okinawa	MOT-16S-1	AB161678	35
	MOT-16S-2	AB161679	15
			Total 50
	MOT-*pmoA*-1	AB089961	2
	MOT-*pmoA*-2	AB089962	3
	MOT-*pmoA*-3	AB089963	1
Mid-Okinawa	MOT-*pmoA*-4	AB089964	3
Trough (MOT)	MOT-*pmoA*-5	AB089965	5
	MOT-*pmoA*-6	AB089966	2
	MOT-*pmoA*-7	AB089967	4
	MOT-*pmoA*-8	AB089968	7
	MOT-*pmoA*-9	AB089969	2
	MOT-*pmoA*-10	AB089970	5
	MOT-*pmoA*-11	AB089971	3
	MOT-*pmoA*-12	AB089972	2
	MOT-*pmoA*-13	AB089973	2
	MOT-*pmoA*-14	AB089974	3
	MOT-*pmoA*-15	AB089975	1
	MOT-*pmoA*-16	AB089976	4
	MOT-*pmoA*-17	AB089977	1
			Total 50
	TAG-16S-1	AB161680	50
			Total 50
TAG	TAG-*pmoA*-1	AB089978	10
Hydrothermal	TAG-*pmoA*-2	AB089979	15
	TAG-*pmoA*-3	AB089980	14
mound (TAG)			
	TAG-*pmoA*-4	AB089981	11
			Total 50

(Sakai et al., 1990b). In contrast, the TAG vent fluid that formed the anhydrite-rich chimneys contains H_2S, methane, and copper (Humphris et al., 1995).

Phylogenetic Analysis of Methanotroph-Specific 16S rDNA

No 16S rDNAs of type II methanotrophs (α-Proteobacteria) were detected by PCR using specific MA221F and universal 1492R primers, despite repeated trials. In contrast, the 16S rDNA of type I (and X) methanotrophs (γ-Proteobacteria) was amplified with the specific primer MG-64F. This result suggests the dominance of type I methanotrophic γ-Proteobacteria in the studied hydrothermal vent samples. Similar results were reported for known methanotrophic symbionts of vent mussels (Distel et al., 1995; Fujiwara et al., 2000).

The PCR products of 16S rDNA were directly sequenced and grouped into 3 OTUs, 2 from the MOT vent plume and one from the TAG chimney. The OTUs TAG-16S-1 and MOT-16S-2 were placed in the branch of type I methanotrophs (Figure 1) and rooted with mussel methanotrophic endosymbionts (Distel et al., 1995; Fujiwara et al., 2000). These OTUs and endosymbiotic sequences form a unique phylogenic subbranch, which is related to the genera *Methylobacter* and *Methylomicrobium.* The known methanotrophic endosymbionts and the free-living methanotrophs may derive from the common ancestor related to *Methylobacter* and *Methylomicrobium* (Hanson and Hanson, 1996; Fujiwara et al., 2000).

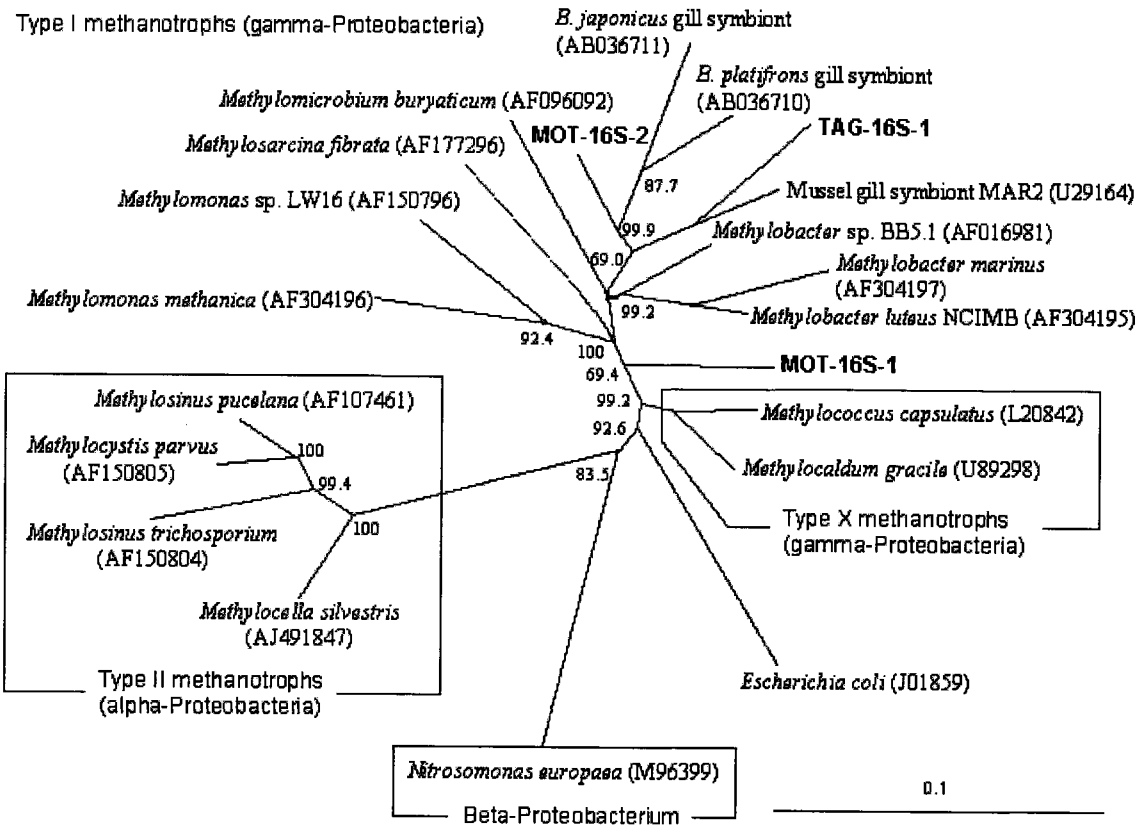

Figure 1. Phylogenetic tree based on 16S rDNA. The tree was constructed by the neighbor-joining method with the Kimura distance. Bootstrap values, calculated from 1000 replicates, are indicated only at major nodes of the tree and expressed as percentages. Scale bar, 0.1 substitutions per site.

The OTU MOT-16S-1 formed a unique phylogenetic lineage near the root of the type I branch and showed maximum nucleotide identity of 93% with the type I methanotroph *Methylobacter luteus* and 85% with the type X methanotroph *Methylococcus capsulatus*. Therefore the MOT-16S-1 may be considered as a novel type I methanotroph.

Diversity of Gene *pmoA* in Deep-Sea Hydrothermal Vent Samples

In comparison with the 16S rDNA, the MOT and TAG samples yielded 17 OPUs (MOT-*pmoA*-1 to -17) and 4 OPUs (TAG-*pmoA*-1 to -4), respectively (Table 1). The high diversity of *pmoA* in the collected samples implies that the gene *pmoA* is a powerful functional molecular tool for studying the diversity of deep-sea methantrophic bacteria. It is notable that the MOT sample also showed high diversity in the *cbbL* gene that encodes the CO_2-fixing enzyme RuBisCO (Elsaied and Naganuma, 2001). The high diversity of *pmoA* and *cbbL* in the MOT sample may be due to the variability in chemical structure of the hydrothermal vent plume, which resulted from the mixture of hydrothermal vent fluids and ambient seawater (Sakai et al., 1990a).

Analysis of Deduced pMOA Amino Acid Sequences and Topologies

The deduced amino acid sequences of pMOA were aligned with those reported from other studies. The deduced OPU amino acid residues in this alignment corresponded to positions 46 to 222 of the pMOA of *Methylococcus capsulatus* (Stainthorpe et al., 1989).

The aligned sequences showed that 6 amino acids (GXWXFW, where X = D, N, or E) from the position 46 to 51 are strongly conserved in all deep-sea pMOA sequences from this and other studies. The amino acid His-168 is conservative in all the aligned sequences except the OPU MOT-*pmoA*-9, where His-168 was substituted by Arg-168. As all known pMOA harbors a polar hydrophilic amino acid at that position, the change from His to Arg may affect

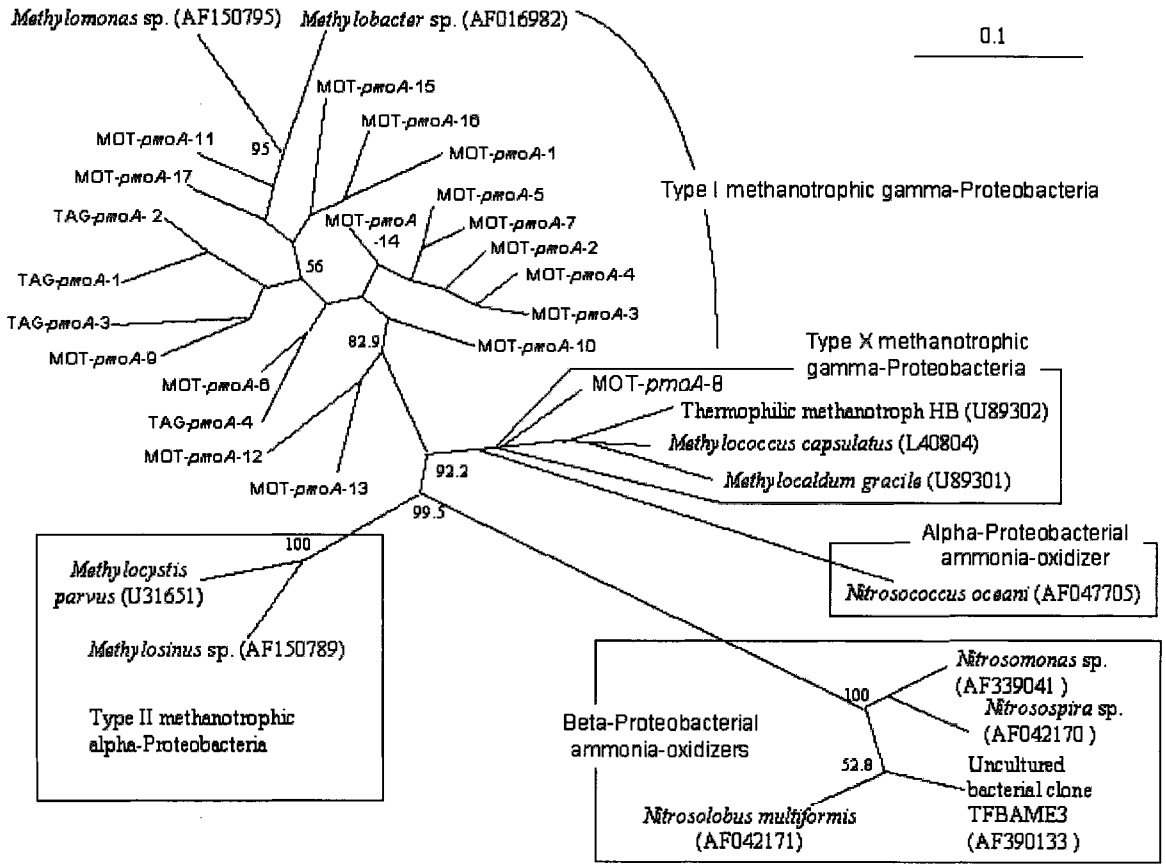

Figure 2. Phylogenetic tree based on the amino acid sequences of the particulate methane monooxygenase subunit A, deduced from the *pmoA* gene sequences. The tree was constructed by the neighbor-joining method with the Kimura distance. Bootstrap values, calculated from 1000 replicates, are indicated only at major nodes of the tree and expressed as percentages. Scale bar, 0.1 substitutions per site.

little. However, there are no physiologic studies regarding this amino acid mutation.

The pMOA deduced from this study has common trans-membrane-spanning regions that are found in most aerobic methanotrophs (Hanson and Hanson, 1996). These regions were localized at amino acid positions 58–80, 85–102, 109–131, and 141–155. The regions generally consist of hydrophobic amino acids and connect with the loops of hydrophilic amino acids exposed to the aqueous environment and bearing the catalytic sites of the enzyme (Semrau et al., 1995).

Phylogenetic Analysis of *pmoA* Sequences

All the OPUs from the hydrothermal vent samples formed the wide range of phylogenetic lineages within the branch of type I and X methanotrophs (γ-Proteobacteria; Figure 2). The OPU MOT-*pmoA*-11 is the most closely related to the known representative type I methanotrophs and showed 90% amino acid identity with *Methylobacter* sp. and 85% with *Methylomonas* sp. It is surprising that the deduced pMOA sequences are remarkably similar among the deep-sea, estuarine, and freshwater methanotrophs (Costello and Lidstrom, 1999; Horz et al., 2001). Salinity and pressure are strong adaptive barriers (Nold and Zwart, 1998), and this result strongly suggests that *pmoA* has been highly conserved through evolution.

The OPU MOT-*pmoA*-8 within the subbranch of γ-Proteobacterial thermophilic methanotrophs showed 80% amino acid identity with that of the type X methanotroph *Methylococcus capsulatus* (Figure 2). Occurrence of type X–like genes such as *pmoA* may thus complement the 16S rDNA-based microfloral survey where the type X–like 16S rDNA was absent (Figure 1). As *M. capsulatus* is a moderate thermophile, the OPU MOT-*pmoA*-8 is also likely to be of thermophilic origin (Trotsenko and Khamelenina, 2002).

Biogeography of Deep-Sea Hydrothermal Vent Methanotrophs

Both 16S rDNA (OTUs) and *pmoA* (OPUs) yielded monophyletic and polyphyletic clusters within the branch of ?-Proteobacterial type I (and X) methanotrophs. Distinct geographic separation of OTUs and OPUs between the 2 sites, MOT and TAG, was not observed, which may reflect the presence of methane in both sites (Sakai et al., 1990b; Humphris et al., 1995).

No 16S rDNA and *pmoA* of type II methanotrophs (α-Proteobacteria) were detected in the studied samples. Similarly, type II–like *pmoA* is absent in the deep-sea sediment off China, although the type II–like gene encoding soluble methane monooxygenase (*smmo*) is present (Wang et al., 2003). This unique biogeographic distribution of deep-sea methanotrophs may reflect the environmentally biased separation of type I and type II methanotrophs (Graham et al., 1993; Prior and Dalton, 1985). For example, the common occurrence of type I–like *pmoA* in the studied hydrothermal vent samples may reflect favored induction of the type I–like *pmoA* expression according to copper ion (Cu^{2+}) or other factors (Sakai et al., 1990b; Nguyen et al., 1994). Our data are based on the analysis of 50 *pmoA* clones each, which may not be too far from the in situ abundance of *pmoA* in each hydrothermal vent site.

Knowledge about the diversity of methane monooxygenase genes in all types of deep-sea methanotrophs is limited and should be increased by more screening of other types of relevant genes, such as *smmo* from a wide range of deep-sea habitats including methanotrophs living on and in the vent-associated animals. This study provided the first step for studying the functional genes that may directly affect methane-oxidizing activity in the methane-rich environments of the deep-sea hydrothermal vent.

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References

Anthony, C. (1982). *The Biochemistry of Methylotrophs*. Academic Press: London, U.K.

Bourne, D., Holmes, A., Iversen, N., and Murrell, J. (2000). Fluorescent oligonucleotide rDNA probes for specific detection of methane-oxidizing bacteria. *FEMS Microbiol Ecol* 31:29–38.

Cavanaugh, C. (1993). Methanotroph-invertebrate symbiosis in the marine environment: ultra structural, biochemical and molecular studies. In: *Microbial Growth on C1 Compounds*, Murrell, J, and Kelly, D (eds.). U.K. Intercept: Andover, pp 315–328.

Costello, A., and Lidstrom, M. (1999). Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. *Appl Environ Microbiol* 65:5066–5074.

Dalton, H. (1991). Structure and mechanism of action of the enzymes involved in methane oxidation. In: *Applications of Enzyme Biotechnology*, Kelly, J.W. (ed.). New York: Plenum Press, pp 55–68.

DiSpirito, A., Gulledge, A., Shiemke, A., Murrell, J., Lidstrom, M., and Krema, C. (1992). Trichloroethylene oxidation by the membrane-associated methane monooxygenase in type I, type II and type X methanotrophs. *Biodegradation* 2:151–164.

Distel, A., and Cavanaugh, C. (1994). Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbiosis in marine bivalves. *J Bacteriol* 176:1932–1938.

Distel, A., Lee, H., and Cavanaugh, C. (1995). Intracelleular coexistence of methano- and thioautotrophic bacteria in a hydrothermal vent mussel. *Proc Natl Acad Sci U S A* 92:9598–9602.

Elsaied, H., and Naganuma, T. (2001). Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* 67:1751–1765.

Felsenstein, J. (1989). PHYLIP-Phylogeny Interference Package (Version 3.2). *Cladistics* 5:164–166.

Fujiwara, Y., Takai, K., Uematsu, K., Tsuchida, S., Hunt, J., and Hashimoto, J. (2000). Phylogenetic characterization of endosymbionts in three hydrothermal vent mussels: influence on host distributions. *Mar Ecol Prog Ser* 208:147–155.

Galchenko, V., and Andreev, L. (1984). Taxonomy of obligatory methylotrophs. In: *Microbial Growth on C1 Compounds*, Crawford, R.L., and Hanson, R. (eds.). Washington, D.C.: American Society for Microbiology, pp 269–281.

Gilbert, B., McDonald, I., Finch, R., Stafford, G., Nielsen, A., and Murrell, J. (2000). Molecular analysis of the *pmo* (particulate methane monooxygenase) operon from two type II methanotrophs. *Appl Environ Microbiol* 66:966–975.

Graham, D., Chaudhary, J., Hanson, R., and Arnold, R. (1993). Factors affecting competition between type I and type II methanotrophs in continuous-flow reactors. *Microb Ecol* 25:1–17.

Gulledge, J., Ahmed, A., Steudler, P., Pomerantz, W., and Cavanaugh, C. (2001). Family- and genus-level 16S rRNA-targeted oligonucleotide probes for ecological studies of methanotrophic bacteria. *Appl Environ Microbiol* 67:4726–4733.

Hanson, R., and Hanson, T. (1996). Methanotrophic bacteria. *Microbiol Rev* 60:439–471.

Hinrichs, K., Hayes, J., Sylva, S., Brewer, P., and DeLong, E. (1999). Methane-consuming archaebacteria in marine sediment. *Nature* 398:802–805.

Holmes, A., Owens, N., and Murrell, J. (1995a). Detection of novel marine methanotrophs using phylogenetic and functional gene probes after methane enrichment. *Microbiology* 141:1947–1955.

Holmes, A., Costello, A., Lidstrom, M., and Murrell, J. (1995b). Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionary related. *FEMS Microbiol Lett* 132:203–208.

Horz, H., Yimga, M., and Liesack, W. (2001). Detection of methanotroph diversity on roots of submerged rice plants by molecular retrieval of *pmoA, mmoX, mxaF* and 16S rRNA and ribosomal DNA, including *pmoA*-based terminal restriction fragment length polymorphism profiling. *Appl Environ Microbiol* 67:4177–4185.

Humphris, S., Herzig, P., Miller, D., Alt, J., Becker, K., Brown, D., Brugmann, G., Chiba, H., Fouquet, Y., Gemmell, J., Guerin, G., Hannington, M., Holm, N., Honnorez, J., Iturrino, G., Knott, R., Ludwig, R., Nakamura, K., Petersen, S., Reysenbach, A., Rona, P., Smith, S., Sturz, A., Tivey, M., and Zhao, X. (1995). The internal structure of an active sea-floor massive sulfide deposit. *Nature* 377:713–716.

Lane, D., Pace, B., Olsen, G., Stahl, D., Sogin, M., and Pace, N. (1985). Rapid determination of 16S ribosomal sequences for phylogenetic analyses. *Proc Natl Acad Sci U S A* 82:6955–6959.

McDonald, I., and Murrell, J. (1997). The particulate methane monooxygenase gene *pmoA* and its uses as a functional gene probe for methanotrophs. *FEMS Microbiol Lett* 156:205–210.

Murrell, J. (1994). Molecular genetics of methane oxidation. *Biodegradation* 5:145–160.

Nguyen, H., Shiemke, A., Jacobs, S., Hales, B., Lidstrom, M., and Chan, S. (1994). The nature of the copper ions in the membranes containing the particulate methane monooxygenase from *Methylococcus capsulatus* (Bath). *J Biol Chem* 269:14995–15005.

Nold, S., and Zwart, G. (1998). Patterns and governing forces in aquatic microbial communities. *Aquat Ecol* 32:17–35.

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

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for constructing phylogenetic tree. *Mol Biol Evol* 4:406–425.

Sakai, H., Gamo, T., Kim, E., Shitashima, K., Yanagisawa, F., and Tsutsumi, M. (1990a). Unique chemistry of the hydrothermal solution in the Mid-Okinawa Trough backarc basin. *Geophys Res Lett* 17:2133–2136.

Sakai, H., Gamo, T., Kim, E., Tsutsumi, M., Tanak, T., Ishibashi, J., Wakita, H., Yamano, M., and Oomori, T. (1990b). Venting of carbon dioxide rich fluid and hydrate formation in Mid-Okinawa Trough Backarc Basin. *Science* 248:1093–1096.

Semrau, J., Chistoserdov, A., Lebron, J., Costello, A., Davagnino, J., Kenna, E., Holmes, A., Finch, R., Murrel, J., and Lidstrom, M. (1995). Particulate methane monooxygenase genes in methanotrophs. *J Bacteriol* 177:3071–3079.

Stainthorpe, A., Murrel, J., Salmond, G., Dalton, H., and Lees, V. (1989). Molecular analysis of methane monooxygenase from *Methylococcus capsulatus* (Bath). *Arch Microbiol* 152:154–159.

Stolyar, S., Costello, A., Peeples, T., and Lidstrom, M. (1999). Role of multiple gene copies in particulate methane monooxygenase activity in the methane-oxidizing bacterium *Methylococcus capsulatus* Bath. *Microbiology* 145:1235–1244.

Trotsenko, Y., and Khamelenina, V. (2002). Biology of extremophilic and extremotolerant methanotrophs. *Arch Microbiol* 177:123–131.

Wang, P., Wang, F., Xu, M., and Xiao, X. (2003). Molecular phylogeny of methylotrophs in a deep-sea sediment from a tropic west Pacific Warm Pool. *FEMS Microbiol Ecol* 1595:1–8.