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Distribution and Phylogenetic Characteristics of the Genes Encoding Enzymes Relevant to Methane Oxidation in Oxygen Minimum Zones of the Eastern Pacific Ocean

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Abstract: A total of 34 pelagic water samples from the upper-, mid-and lower-Oxygen Minimum Zones (OMZs) in the eastern Pacific Ocean were collected. Particles suspended therein were captured by 0.2 μm pore size filters to extract bulk genomic DNA for PCR amplification of the genes relevant to methane oxidation. The genes encoding particulate methane monooxygenase and its relative enzyme ammonia monooxygenase (β), *pmoA* and *amoA*(β), respectively, were amplified and analyzed. Five OMZ samples (from four sites) showed amplification of *pmoA*. This finding provide the bases for consideration that pelagic methane oxidation may occur in OMZs. Interestingly, one *pmoA* sequence was identical (based on amino acids) to *pmoA* of the methanotrophic endosymbiont.

Key words: Methane monooxygenase, ammonia monooxygenase, *pmoA*, *amoA*, methanotroph

INTRODUCTION

Oxygen Minimum Zones (OMZs) are the pelagic regions characterized by dissolved oxygen concentration as low as <0.5 mL L^{-1} (<22 μM, or <7.5% of saturation; Levin, 2003). Large-scale OMZs are found in the southeast Atlantic off West Africa, the northern Indian Ocean and the eastern Pacific Ocean (Helly and Levin, 2004) and hypoxic to anoxic conditions are maintained in the middle parts of OMZs, namely OMZ cores. Denitrification is a typical anaerobic biogeochemical process in OMZs (Codispoti *et al*., 2005; Deutsch *et al*., 2007) and releases nitrogen suboxide (N_2O) that contributes to global warming along with carbon dioxide and methane (Houghton *et al*., 2001).

Occurrence of a methane pool has been reported in the eastern tropical North Pacific Ocean (Burke *et al*., 1983; Sansone *et al*., 2001; Sansone *et al*., 2004). The methane pool is a water column region characterized by >10 nM methane, centered at water depths of 250-650 m with >500 m thickness and >1000 km horizontal extension in the OMZ and contributes to methane release into atmosphere. Similar methane pool is proposed for the off-Chile OMZ in the eastern South Pacific Ocean (Sansone *et al*., 2001). Stable carbon isotope analysis suggested that the methane in OMZs are subject to aerobic microbial oxidation (Sansone *et al*., 2001; Sansone *et al*., 2004).

The microbial agents that oxidize methane to obtain metabolic energy are also known to assimilate part of methane as the carbon source via either the ribulose monophosphate pathway (type I) or serine cycle (type II) (Hanson and Hanson, 1996) and thus termed methanotrophs. The type I methanotrophs possess the enzymes, particulate methane monooxygenase (pMMO), while the

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type II methanotrophs possess both pMMO and soluble methane monooxygenase (sMMO). Active centers of pMMO and sMMO are encoded by the genes, *pmoA* and *mmoX*, respectively. Thus, *pmoA* and *mmoX* are often used as genetic markers to study diversity and distribution of methanotrophs in natural environments (Dumont and Murrell, 2005). The enzyme, ammonia monooxygenase (AMO), is also responsible for aerobic methane oxidation, despite lower activity than pMMO and sMMO (Bedard and Knowles, 1989). The gene *amoA* encodes the AMO active center, with two subclasses of *amoA*(β) and *amoA*(γ) according to their carriers β- and γ-Proteobacteria, respectively (Ward and O'Mullan, 2005).

Therefore, four genes, *pmoA*, *mmoX*, *amoA*(β) and *amoA*(γ), are available as genetic markers. However, these genetic markers have not been applied to detect and characterize the distribution and diversity of methane-oxidizing enzymes in OMZs. This study aims at qualitative cataloging of *pmoA*, *mmoX*, *amoA*(β) and *amoA*(γ) by analysis of the PCR-amplified clones and reports the occurrence and diversity of these genes in the OMZs of eastern Pacific Ocean, covering from eastern tropical North Pacific Ocean to off-Peru South Pacific Ocean.

MATERIALS AND METHODS

Collection of Water Samples and Suspended Particles

Water samples from OMZs in the eastern Pacific Ocean were collected at the sites HY01 to HY10A and H15A (Table 1) during the Legs 3 and 4 of the KH-03 cruise (June to August 2003) by RV *Hakuhomaru*, University of Tokyo (currently operated by Japan Agency for Marine-Earth Science and Technology). The Dissolved Oxygen (DO) profiles (Fig. 1) were obtained at each site by CTD equipped with a DO sensor. The sampled-water depth was determined by DO, which is corresponding to approximately 50% of surface water and minima. In this study, these samples were regarded as the representative water of OMZs. Figure 2 shows the vertical section of dissolved oxygen (mL L^{-1}) along 95°W (8°N to 8°S, which correspond to the site HY03-HY09). The site 15A showed little development of OMZ (Fig. 1) and was thus regarded as the negative control. Water samples were collected with Niskin bottles and three- to five-liter aliquots were filtered through Sterivex filters (pore size, 0.22 μm; Millipore Corp., Bedford, Massachusetts) to capture DNA-containing particles suspended therein. The used Sterivex filters were washed with 1.8 mL of STE buffer (20% sucrose, 50 mM EDTA, 50 mM Tris-HCl, pH 8.0) and kept frozen at -20°C on board until DNA extraction in the onshore laboratory, according to Somerville *et al*. (1989).

Extraction and Amplification of Bulk Genomic DNA

The sterivex filters were added with 1.8 mL of SET buffer and 62 μL of lysozyme solution (5 mg mL^{-1} in TE buffer containing 10 mM Tris-HCl, 1 mM EDTA and 10 mM NaCl, pH 8.0) and incubated on ice for 15 min, to extract bulk genomic DNAs. The primary lysates were further incubated with 16 μL of 25% SDS at room temperature for 1 h. Finally the lysates were incubated with 50 μL of proteinase K (20 mg mL^{-1}) at room temperature for 3 h.

The lysates were separately collected from the Sterivex filter cassettes using sterilized syringes and the residual lysates were collected by adding 1 mL each of SET buffer and then a collection with fresh syringes. The original and residual lysates from a Sterivex filter were mixed and used for DNA precipitation by the phenol-chlorofom-isopropanol method. The DNA precipitates were immediately used for amplification of the genetic marker *pmoA*, *mmoX*, *amoA*(β) and *amoA*(γ) sequences.

PCR Amplification of Marker Gene Sequences

The amplified bulk genomic DNAs were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, Calif.) and prepared at 70 ng μL^{-1} each, which were tested by PCR with the primers

Table 1: Sites of OMZ water sample collection, sample codes, physico-chemical parameters and detected genetic markers

Site	Latitude, longitude	Water depth (m)	Sample code	Sampled depth (m)	Water temperature (°C)	Salinity (psu)	Dissolved oxygen (mL L^{-1})	Detected genetic marker
HY01	20°0.09'N	5315	1 h	271	12.6	34.2	2.98	
	140°0.41'W		1 m	596	6.8	34.4	0.36	
HY02	16°30.92'N	4221	2 h	137	15.6	34.3	2.09	
	123°0.22'W		2 m	198	12.4	34.7	0.09	*pmoA*
HY03	8°2.17'N	3671	3 h	161	12.2	34.8	0.45	
	94°57.18'W		3 m	371	9.7	34.7	0.14	
			3 c	497	8.1	34.6	0.14	*pmoA*
HY04	4°2.07'N	3578	4 h	78	17.2	34.7	1.62	
	95°2.84'W		4 m	329	10.6	34.7	0.52	
			4 c	419	8.9	34.6	0.12	
HY05	2°1.28'N	2848	5 h	83	16.6	34.9	2.16	
	95°29.79'W		5 m	323	11.2	34.7	0.28	
			5 c	383	9.8	34.7	0.23	
HY06	0°2.18'N	3247	6 h	192	13.3	34.9	1.99	
	95°26.50'W		6 m	376	10.5	34.7	0.20	
			6 c	409	9.9	34.7	0.22	
HY07	2°1.76'S	3325	7 h	67	16.0	35.0	1.99	
	94°59.94'W		7 m	316	11.4	34.8	0.16	
			7 c	336	11.0	34.8	0.16	
HY08	3°58.11S9	3622	8 h	50	20.9	35.1	3.54	
	5°0.31W		8 m	351	11.1	34.8	0.15	
			8 c	397	10.0	34.7	0.11	
HY08B	5°57.30'S	3828	8 Bh	96	17.2	35.2	1.93	*amoA*(β)
	94°55.37'W		8 Bm	331	11.1	34.8	0.23	
			8 Bc	439	9.3	34.7	0.13	*pmoA*
HY09	7°58.71'S9	3870	9 h	83	17.8	35.1	1.75	*amoA*(β)
	5°0.66'W		9 m	129	13.3	34.9	0.12	
			9 c	329	10.5	34.7	0.13	
HY10A	8°0.39'S	4157	10 Ah	28	20.2	35.2	3.47	
	85°49.69'W		10 Am	282	11.4	34.8	0.13	*pmoA*
			10 Ac	398	9.6	34.7	0.12	*pmoA*
HY15A	25°0.70'S	3042	15 Ah	447	8.3	34.3	3.10	
	116°0.02'W		15 Am	991	4.1	34.4	3.02	
			15 Ac	1090	3.6	34.4	3.04	

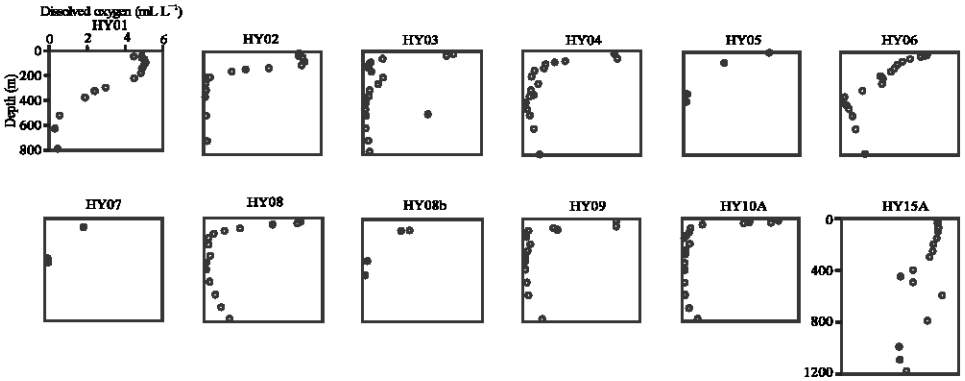

Fig. 1: Dissolved oxygen profile at each site. The black circles show the sampled-water depth

for bacterial 16S rRNA gene (Lane, 1991). Then the bulk DNAs were used to amplify about 500 bp each of the *pmoA* (full length, 744 bp), *mmoX* (1584 bp), *amoA*(β) (831 bp) and *amoA*(γ) (744 bp) sequences using ExTaq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan) with a TaKaRa Cycler

Table 2: PCR primer sets and conditions for amplification of the genetic markers, *pmoA*, *mmoX*, *amoA*(β) and *amoA*(γ) sequences

Genetic marker (Ref)		Forward (F) and reverse (R) primer set	Thermal cycle condition			
			Denaturation	Annealing	Extension	Cycles
pmoA	F	5'-GGNGACTGGGACTTCTGG-3'	92°C	55°C	72°C	
(a)	R	5'-CCGGMGCAACGTCYTTACC-3'	60 sec	90 sec	60 sec	25
mmoX	F	5'-CGGTCCGCTGTGGAAGGGCATGAAGCGCGT-3'	94°C	55°C	72°C	
(b)	R	5'-GGCTCGACCTTGAACTTGGAGCCATACTCG-3'	60 sec	60 sec	60 sec	30
amoA(β)	F	5'-GGGGTTTCTACTGGTGGT-3'	94°C	55°C	72°C	
(c)	R	5'-CCCCTCKGSAAAGCCTTCTTC-3	15 sec	20 sec	40 sec	30
amoA(γ)	F	5'-GGTGAGTGGGYTAACMG-3'	94°C	48°C	72°C	
(d)	R	5'-GCTAGCCACTTTCTGG-3'	15 sec	20 sec	40 sec	30

(a), Costello and Lidstrom (1999) (b), Miguez *et al.* (1997) (c), Rotthauwe *et al.* (1997) and (d) Purkhold *et al.* (2000)

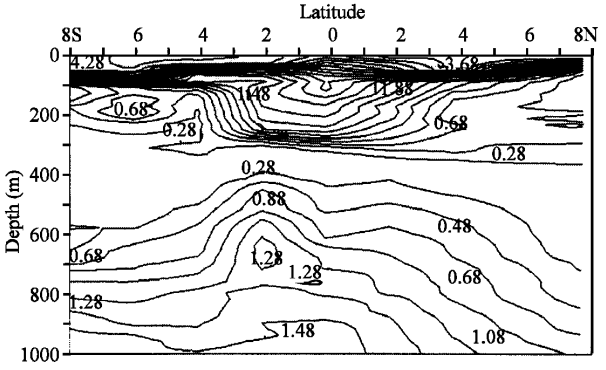

Fig. 2: Vertical section of dissolved oxygen (mL L^{-1}) along 95°W

PERSONAL TP240, according to the PCR primers and conditions (Costello and Lidstrom, 1999; Miguez *et al.*, 1997; Purkhold *et al.*, 2000; Rotthauwe *et al.*, 1997) listed in Table 2.

Cloning, Sequencingand Molecular Analyses

The PCR products of the expected sizes were excised after agarose gel electrophoresis, purified with the QIAquick PCR Purification Kit and cloned using the TOPO Cloning Kit with One Shot TOP10 *E. coli* (Invitrogen Corp., Carlsbad, Calif.). Twenty four transformants per sample, if PCR-positive, were randomly collected and sequenced bi-directionally by the dideoxy method (Sanger *et al.*, 1977) on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, Calif.). Retrieved sequences were searched for homology based on both nucleotides and amino acids by FASTA at the DNA Data Bank of Japan (DDBJ; www.ddbj.hig.ac.jp). Sequences non-homologous to target genes were excluded from further analyses.

The sequences having >97% nucleotide similarities were grouped into an operational unit. The most equidistant sequence within a unit was chosen to represent the unit and the representative sequences were deposited to DDBJ under the accession numbers AB276025 to AB276029. Each representative was converted to amino acid sequences to construct a phylogenetic tree along with known closely related sequences using the MEGA3 program (Kumar *et al.*, 2004).

The sequences were checked for chimeras by bisecting and drawing two sub-phylogenetic trees from the bisects of each sequence. The sequences that showed different topologies among the two sub-trees were regarded as chimeric and removed from analyses. Transmembrane-spanning regions and topology of the deduced pMMO and AMO proteins were estimated using the TMHMM tools (br.expasy.org/tools/).

RESULTS AND DISCUSSION

PCR Clone Libraries of Amplified *pmoA* and *amoA*(β) Sequences

A total of 90 *pmoA* and 45 *amoA*(β) PCR clones were obtained from five and two samples, respectively, out of total 34 samples, were obtained (Table 3), while *mmoX* and *amoA*(γ) sequences were not amplified by PCR despite repeated trials with the standard (Table 2) and modified thermal cycles.

The chimera-checked clones having≥ 97% nucleotide sequence similarities were grouped into an operational *pmoA* or *amoA*(β) units, OPU and OAU, respectively, which represent the equidistant (least deviated) sequences of component clones. As a result, four OPUs (OPU1 to OPU4, in the order of clone numbers shown in Table 3) and one OAU (OAU1) were formed. The four OPUs showed nucleotide similarities of 66.0 to 94.6%.

The *pmoA* and *amoA*(β) sequences yielded putative transmembrane regions of the corresponding enzymes pMMO and AMO, respectively and the numbers and lengths of putative transmembrane regions were compared with known counterparts of *Methylococcus capsulatus* and *Nitrosomonas europaea* (Table 4). The OPU1 to OPU4 encoded 169 amino acids, in which four transmembrane regions were identified at the amino acid positions at 13-35, 40-57, 64-86 and 96-118 (OPU1) or 90-112 (OPU2 to OPU4; identical to that of *M. capsulatus*). Similarly, the OAU1 encoded 163 amino acids, in which three transmembrane regions were identified at the amino acid positions at 10-32, 37-59, 107-129, nearly identical to that of *N. europaea.*

Thus, the obtained OPU and OAU sequences were highly likely corresponding to *pmoA* and *amoA*(β), respectively and contributed to archiving of marine *pmoA* and *amoA* genes that have been underrated. The *pmoA* sequences in particular are the first from the vast oceanic water column that is the global largest habitat.

Phylogenetic Analysis of Amplified pmoA and amoA(β) Sequences

The FASTA nucleotide homology-search showed that the most closely related at a 73.5% (nucleotides) similarity to the *pmoA* of the thermophilic methanotroph, *Methylocaldum szegediense* OR2 (U89303; Bodrossy *et al*., 1997), which was originally isolated from a Hungarian geothermal fluid and grows up to 62°C.

It should be noted that the OPU2 is the most closely related at a 99.8% similarity to the *pmoA* of the methanotrophic symbiont of the vent mussel *Bathymodiolus* sp. (AB062137). The high score of boot strap values supports the phylogenetic positioning of OPU2 (Fig. 3). The mussel specimens were collected from a 1035 m deep hydrothermal vent in the mid-Okinawa Trough, western North

Table 3: Distribution of retrieved *pmoA* and *amoA*(β) clones in seven OMZ water samples (from five sites) of the eastern Pacific Ocean. Sample codes are listed in Table 1, The number of representative sequence is given in parentheses

	Operational *pmoA*/*amoA*(β) unit (OPU/OAU) (Serial accession numbers AB276025- AB276029)					
Sample code	OPU1	OPU2	OPU3	OPU4	OAU1	Clone number
2 m	12 (6)					12
3 c	15 (3)	5 (3)				20
8 Bh					21 (7)	21
8 Bc	16					16
9 h					24 (12)	24
10 Am		21 (5)				21
10 Ac	4		11 (4)	6 (1)		21
Clone number	47	26	11	6	45	Total 135 clones

Table 4: Numbers of amino acid residues, numbers of transmembrane (TM) regions and TM amino acid positions inferred from the *pmoA* and *amoA*(β) sequences of the OMZ clones (this study) and the selected species, *Methylococcus capsulatus* and *Nitrosomonas europaea*

Target gene	OMZ clone or selected species	Amino acid residues	No. of TM regions	TM amino acid positions
pmoA	OPU1,	169	4	13-35, 40-57, 64-86, 96-118
	OPU2,OPU3,OPU4	169	4	13-35, 40-57, 64-86, 90-112
	M. capsulatus	169 (full 247)	4	13-35, 40-57, 64-86, 90-112
amoA(β)	OAU1	163	3	10-32, 37-59, 107-129
	N. europaea	163 (full 276)	3	10-32, 37-59, 108-130

Pacific Ocean and known to harbor a unique endosymbiont, namely, type X methanotroph, that possesses the genes encoding both methanotrophic and autotrophic enzymes, *pmoA* and *cbbL*, simultaneously (Elsaied and Naganuma, 2001; Elsaied *et al*., 2006).

The OPU3 and OPU4 were most closely but only weakly related at 78.3 and 76.6% similarities, respectively, to the *pmoA* of *Methylomicrobium* sp. NI (AB253367), a marine methanotroph that notably possesses particulate and soluble methane monooxygenase genes simultaneously (Nakamura *et al*., unpublished).

As to the deduced amino acids, FASTA resulted in the closest homology of OPU1 at an 81.5% similarity to an environmental clone (Q75NB8) from the 650 m deep methane seep sediment in the western North Pacific Ocean (Inagaki *et al*., 2004); OPU2 at 100% identity to an environmental clone (Q8KZJ5) from hydrothermal chimney fragments of the TAG mound, 3655 m deep, Mid-Atlantic Ridge (Elsaied *et al*., 2004) and OPU3 and OPU4 both at a 93.9% similarity to an environmental clone (Q19PD7) from the 540 m deep hydrocarbon seep sediment in the Gulf of Mexico (Yan *et al*., 2006).

As mentioned above, the OPU2 is also showed the 100% identity (amino acids; 99.5% similarity based on nucleotides) to the *pmoA* environmental clone from the TAG hydrothermal mound, Mid-Atlantic Ridge (Q8KZJ5; Elasied *et al*., 2004). The shortest distance of about 15000 km lies between the OPU2-positive sites and the mid-Okinawa Trough; about 6000 km between the OPU2-positive sites and the TAG mound and about 14500 km between the mid-Okinawa Trough and the TAG mounds. It should be noted that the OPU2 was the second abundant operational *pmoA* unit (Table 3). Therefore, the OPU2 may be widely shared (via lateral gene transfer) among diverse methanotrophs or represent a cosmopolitan but low O_2-adapted methanotroph in the global oceanic regimes.

The nucleotide sequence of the *amoA*(β) OAU1 was the most closely related at a 86.9% similarity to the *amoA*(β) of the environmental isolate bacterium *amoA*.12.V-frei.kultur (AY795821) from the inland saline soil of Hannoversches Wendl and Schreyahn, Germany (unpublished). The deduced amino acid sequence of the OAU1 was the most closely related at 99.3% identity to the environmental clone (Q66UX2) from the Plum Island Sound estuary sediment, Massachusetts (Bernhard *et al*., 2005). No marine *amoA*(β) counterparts were related to the OAU1 at >90% similarities.

Distribution of pmoA and amoA(β) in Eastern Pacific Ocean OMZs

Eastern Pacific OMZs are regarded to host methane pools (Sansone *et al*., 2004), whatever the sources and extents are and thus it is implied that peripheries (i.e., redox boundaries) of the OMZs may correspond to the sites of aerobic methane oxidation. Methane oxidation in water column should require a redox boundary in which both methane and molecular oxygen exist and such redox gradients are likely formed in the OMZ peripheries or in association with OMZ-trapped marine snows, for example. Five OMZ waters (0.09-0.14 mL L^{-1} O_2) yielded PCR amplification of *pmoA* sequences, which suggests that aerobic methane oxidation occurs in these anoxic waters. In other words, distribution of *pmoA* may be influenced by the occurrence of methane pools that are formed in association with OMZs. The sites of two *pmoA*-positive waters, HY02 and HY03, corresponded to

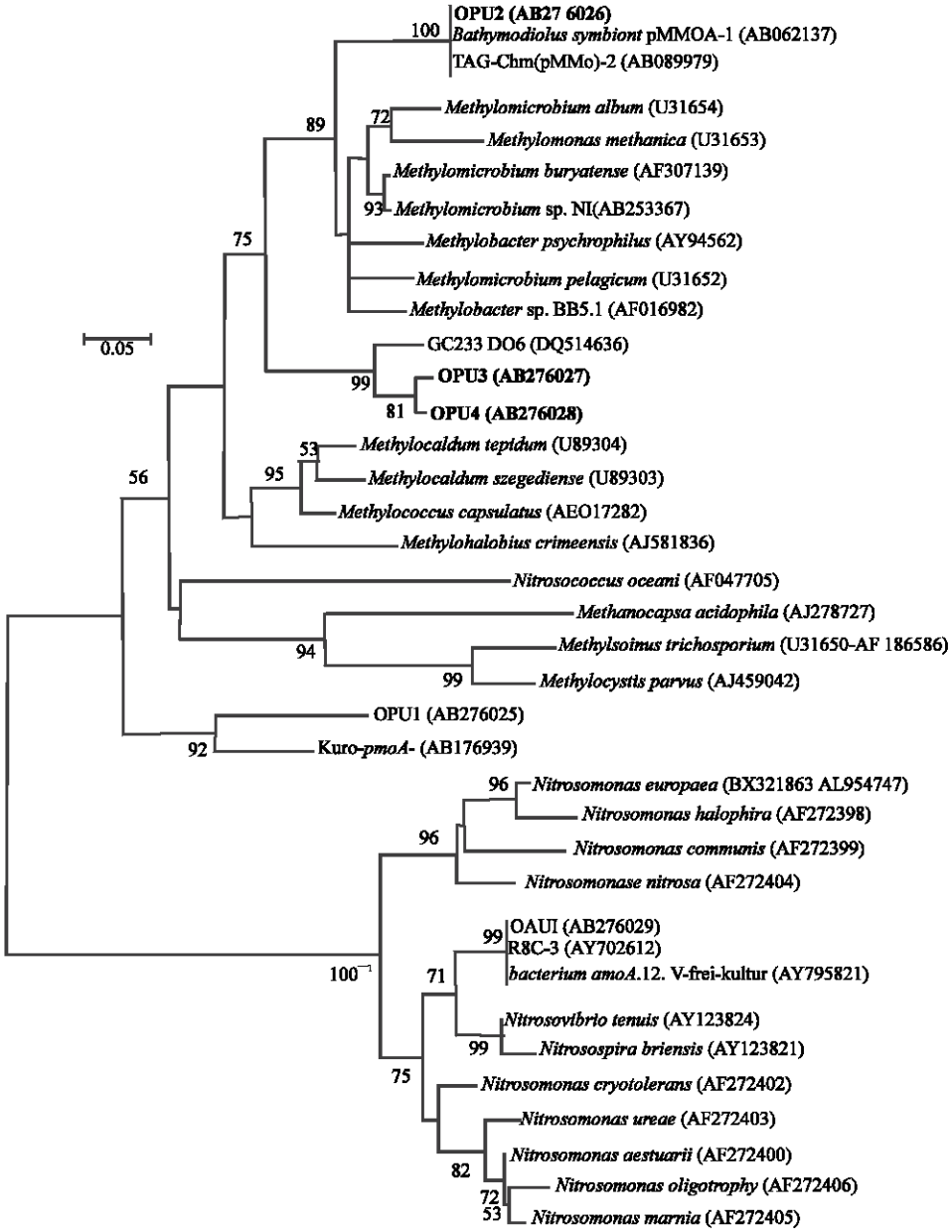

Fig. 3: The neighbor-joining phylogenetic tree based on the deduced *pmoA* and *amoA* amino acid sequences (102 amino acids) of the OMZ clones and cultured species. Bootstrap values (percentage of 1000 replications) greater than 50 are shown on or horizontally to nodes. Scale bar, 0.05 substitutions per site

the periphery of the eastern tropical North Pacific methane pool observed in 1983 and 2001 (Burke *et al*., 1983; Sansone *et al*., 2001) and the sites of HY08B and HY10A were possibly located within the off-Peru methane pool (Sansone *et al*., 2001).

The observed inconsistence of *pmoA* distribution and DO concentration may be ascribed to: 1) time-lags between the formation/disappearance of methane-oxidizing redox conditions and corresponding microflora and/or 2) involvement of other parameters such as concentration of copper that is required for the expression of pMMO enzymatic activity (Hanson and Hanson, 1996).

In contrast, the equatorial sites HY04 to HY08, where OMZs (with <0.2 mL L^{-1} O_2) were likely less developed (Fig. 2), yielded no amplification of *pmoA*. Equatorial water columns, particularly >250 m deep, have higher dissolved oxygen concentrations and less developed OMZs (Helly and Levin, 2004), due to migration of the O_2-rich eastward-moving Equatorial Undercurrent from 150°E to off-Ecuador (Tsuchiya, 1968). The O_2-rich water mass serves as capping and depressing the upper OMZ boundaries in the equatorial (5° N to 5° S, Fig. 2) eastern Pacific Ocean (Helly and Levin., 2004) and thus may have hindered the formation of m ethane-oxidizing conditions and thus occurrence of *pmoA* there.

The *amoA* sequence, an additional genetic marker for potential aerobic methane oxidation, was detected in two shallower hypoxic, not anoxic, water samples (HY08B-8Bh at 96 m deep with 1.93 mL L^{-1} O_2 and HY09-9h at 83 m deep with 1.75 mL L^{-1} O_2). This may suggest that the *amoA*-encoded enzyme, ammonia monooxygenase (AMO) catalyzes aerobic methane oxidation in these non-mid-OMZ waters with different enzymological featuers such as sensitivity to O_2, substrate affinity K_m and velocity V_{max}, or that AMO catalyzes oxidation of ammonia rather than methane to yield nitrite. Part of the regenerated nitrate via ammonia oxidation might be recycled by nitrate reduction or denitrification that is active in the eastern Pacific Ocean (Zehr and Ward, 2002).

CONCLUSIONS

This study displayed the occurrence and diversity of pelagic *pmoA* and *amoA*(β), the genes coding for enzymes probably involved in pelagic methane oxidation in the OMZs of eastern Pacific Ocean. In future study, quantitative analysis using messenger RNA (mRNA) instead of DNA may allow us to determine whether these gene sequences are actually from active, predominant cells or not, although *in situ* or on-board detection of mRNA requires more expertise and sophisticated set-ups with thorough anti-contamination measures.

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REFERENCES

Bedard, C. and R. Knowles, 1989. Physiology, biochemistry and specific inhibitors of CH_4, NH_4 and CO oxidation by methanotrophs and nitrifiers. Microbiol. Rev., 53: 68-84.

Bernhard, A.E., T. Donn, A.E. Giblin and D.A. Stahl, 2005. Loss of diversity of ammonia-oxidizing bacteria correlates with increasing salinity in an estuary system. Environ. Microbiol., 7: 1289-1297.

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

Burke, R.A., D.F. Reid, J.M. Brooks and D.M. Lavoie, 1983. Upper water column methane geochemistry in the eastern tropical North Pacific. Limnol. Oceanogr., 28: 19-32.

Codispoti, L.A., T. Yoshinari and A.H. Devol, 2005. Suboxic Respiration in the Oceanic Water Column. In: Respiration in Aquatic Ecosystems, Del Giorgio, P.A. and P.J. LeB. Williams (Eds.), Blackwell, Oxford, pp: 225-247.

Costello, A.M. and M.E. Lidstrom, 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Applied Environ. Microbiol., 65: 5066-5074.

Deutsch, C., J.L. Sarmiento, D.M. Sigman, N. Gruber and J.P. Dunne, 2007. Spatial coupling of nitrogen inputs and losses in the ocean. Nature, 445: 163-167.

Dumont, M.G. and J.C. Murrell, 2005. Community-Level Analysis: Key Genes of Aerobic Methane Oxidation. In: Methods in Enzymology Leadbetter, J. (Ed.), Academic Press, New York, 397: 413-427.

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

Elsaied, H.E., T. Hayashi and T. Naganuma, 2004. Molecular analysis of deep-sea hydrothermal vent aerobic methanotrophs by targeting the genes of 16S rRNA and particulate methane monooxygenase. Mar. Biotechnol., 6: 503-509.

Elsaied, H. E., R. Kaneko and T. Naganuma, 2006. Molecular characterization of a deep-sea methanotrophic mussel symbiont that carries a RuBisCO gene. Mar. Biotechnol., 8: 511-520.

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

Helly, J. and L.A. Levin, 2004. Global distribution of naturally occurring marine hypoxia on continental margins. Deep-Sea Res. I., 51: 1159-1168.

Houghton, J.T., Y. Ding, D.J. Griggs, M. Noguer, P.J. van der Linden and D. Xiaosu, 2001. IPCC climate change 2001: The scientific basis. Contribution of working group I to the third assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge University Press, Cambridge, United Kingdom.

Inagaki, F., U. Tsunogai, M. Suzuki, A. Kosaka and H. Machiyama *et al.*, 2004. Characterization of C1-metabolizing prokaryotic communities in methane seep habitats at the Kuroshima Knoll, southern Ryukyu Arc, by analyzing *pmoA*, *mmoX*, *mxaF*, *mcrA* and 16S rRNA Genes. Applied Environ. Microbiol., 70: 7445-7455.

Kumar, S., K. Tamura and M. Nei, 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief. Bioinform., 5: 150-163.

Lane, D.J., 1991. 16S/23S rRNA Sequencing. In: Nucleic Acid Techniques in Bacterial Systematics. Stackebrandt, E. and M. Goodfellow, (Eds.), J. Wiley and Sons, New York, pp: 115-175.

Levin, L.A., 2003. Oxygen minimum zone benthos: Adaptation and community response to hypoxia. Oceanogr. Mar. Biol. Ann. Rev., 41: 1-45.

Miguez, C.B., D. Bourque, J.A. Sealy, C.W. Greer and D. Groleau, 1997. Detection and isolation of methanotrophic bacteria possessing Soluble Methane Monooxygenase (sMMO) genes using the polymerase chain reaction (PCR). Microb. Ecol., 33: 21-31.

Purkhold, U., A. Pommerening-Röser, S. Juretschko, M.C. Schmid, H.P. Koops and M. Wagner, 2000. Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and *amoA* sequence analysis: Implications for molecular diversity surveys. Applied Environ. Microbiol., 66: 5368-5382.

Rotthauwe, J.H., K.P. Witzel and W. Liesack, 1997. The ammonia monooxygenase structural gene *amoA* as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing populations. Applied Environ. Microbiol., 63: 4704-4712.

Sanger, F., S. Nicklen and A.R. Coulson, 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA., 74: 5463-5467.

Sansone, F.J., B.N. Popp, A. Gasc, T.M. Rust and A.W. Graham, 2001. Highly elevated methane in the eastern tropical North Pacific and associated isotopically enriched fluxes to the atmosphere. Geophys. Res. Lett., 28: 4567-4570.

Sansone, F.J., A.W. Graham and W.M. Berelson, 2004. Methane along the western Mexican margin. Limnol. Oceanogr., 49: 2242-2255.

Somerville, C.C., I.T. Knight, W.L. Straube and R.R. Colwell, 1989. Simple, rapid method for direct isolation of nucleic acids from aquatic environments. Applied Environ. Microbiol., 55: 548-554.

Tsuchiya, M., 1968. Upper Waters of the Intertropical Pacific Ocean. The Johns Hopkins Press, Baltimore, pp: 50.

Ward, B.B. and G.D.O. Mullan, 2005. Community-Level Analysis: Genetic and Biogeochemical Approaches to Investigate Community Composition and Function in Aerobic Ammonia Oxidation. In: Methods in Enzymology, Vol. 397, Leadbetter, J. (Ed.), Academic Press, New York, pp: 395-413.

Yan, T., Q. Ye, J. Zhou and C.L. Zhang, 2006. Diversity of functional genes for methanotrophs in sediments associated with gas hydrates and hydrocarbon seeps in the Gulf of Mexico. FEMS Microbiol. Ecol., 57: 251-259.

Zehr, J.P. and B.B.Ward, 2002. Nitrogen cycling in the ocean: New perspectives on processes and paradigms. Applied Environ. Microbiol., 68: 1015-1024.