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# Molecular Diversity of the Genes Encoding Ammonia Monooxygenase and Particulate Methane Monooxygenase from Deep-sea Sediments

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**Abstract:** We have profiled the diversity of functional genes encoding diagnostic enzymes of aerobic ammonia and methane oxidation in the Nankai Trough sediments. Bulk DNAs were extracted from 79 deep-sea sediment core samples, off-central Japan. Total 429 clones of the genes encoding ammonia monooxygenase, *amoA*(β) and particulate methane monooxygenase, *pmoA*, yielded ten and two operational units, respectively. Patchy distributions of the clones were uncorrelated to known geological and geochemical features of the sediments.

**Key words:** Nitrification, ammonia oxidation, methane oxidation, Nankai Trough

## INTRODUCTION

Ammonia oxidation, or nitrification, is a major pathway of biogeochemical cycling of nitrogen (Capone, 2000). Nitrification consists of serial oxidations of ammonia to hydroxylamine by ammonia monooxygenase, to nitrite and to nitrate (Ward, 2000). These oxidations support chemoautotrophy of nitrifying bacteria (Kowalchuk and Stephen, 2001) such as betaproteobacterial *Nitrosomonas* and *Nitrospira* species and gammaproteobacterial *Nitrosococcus halophilus* and *N. oceani* (Ward and O'Mullan, 2002).

Environmental bacterial diversities and community structures are often studied by non-culture-dependent methods based on 16S rRNA gene sequence analyses. However, diagnostic and phylogenetic analyses of ammonia-oxidizing bacteria often employ PCR targeting ammonia monooxygenase genes, *amoA*, rather than 16S rRNA genes due to greater sequence divergence (McTavish *et al*., 1993; Purkhold *et al*., 2000; Rotthauwe *et al*., 1997; Ward and O'Mullan, 2005). Previous study showed the dominance of betaproteobacterial *amoA*(β) over gammaproteobacterial *amoA*(γ) in shallow marine sediments (Nold *et al*., 2000). Contrary, little has been known for diversity of deep-sea *amoA*.

Methane monooxygenase catalyzes ammonia oxidation and *vice versa* (Bedard and Knowles, 1989), as molecular structures of methane and ammonia are closely similar. It is thus likely that methane-oxidizing bacteria contribute to ammonia oxidation in nature and *vice versa* (Bedard and Knowles, 1989; Bodelier and Frenzel, 1999; Erwin *et al*., 2005; Jiang and Bakken, 1999; Roy and Knowles, 1994; Ward, 1990). Methane-oxidizing bacteria diversity is often studied using the functional genes *pmoA* and *mmoX*, encoding subunits of methane monooxygenases (Hanson and Hanson, 1996).

We have characterized the genes in relevance to ammonia oxidation, bacterial *amoA* and *pmoA*, in deep-sea sediment cores to catalogue their phylogenetic diversity and distribution. Archaeal *amo*A in marine water columns and sediments (Wuchter *et al*., 2006) was not targeted, because its phylogenetic and functional relationship to *pmoA* was unclear.

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## MATERIALS AND METHODS

### Sample Collection

Ten sediment cores were collected from four areas in the Nankai Trough, off-central Japan, using a 5 m-long piston corer with a dispensable acryl inner tube (Table 1). Sediment features such as porosity, grain size (clay, silt to sand), interstitial sulfate concentration were recorded (Tanahashi *et al*., unpublished). The sediment cores were longitudinally halved and samples were collected from 79 different layers using sterile tip-cut 5-mL syringes (Terumo Corp., Tokyo, Japan). The mud-filled syringes were plugged and frozen at -20°C onboard.

Table 1: Distribution of retrieved *amoA*(β) clones in 10 sediment core samples (79 sections) from the Nankai Trough seafloor

| Core code Latitude Longitude Water depth | Section (cm below seafloor) | Operational *amoA*(β) unit (OAU) [Accession number AB261611-AB261620] | | | | | | | | | | Sectional clone number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | |
| BO-03-PC01 | 15-16 | 9 | 3 | | | | | | | | | 12 |
| 33°45.74'N | 40-41 | | | 9 | 1 | | | | | | | 10 |
| 136°27.60'E | 55-56 | 10 | | | | | | | | | | 10 |
| 2057 m | 72-73 | 12 | | | | | | | | | | 12 |
| | 94-95 | 9 | | 2 | | | | | | | | 11 |
| | 108-109 | 8 | | 3 | | | | | | | | 11 |
| | 132-133 | 8 | | 1 | | | | | | | | 9 |
| | 152-153 | 5 | | 1 | | | | | | | | 6 |
| | 186-187 | 6 | | | | | | | | | | 6 |
| | 192-193 | 11 | | | | | | | | | | 11 |
| BO03-PC02 | 20-21 * | 5 | 3 | | 2 | | | 1 | | | | 11 |
| 33°50.00'N | 37-38 | 8 | | | 1 | | 1 | | 1 | 1 | | 12 |
| 136°25.92'E | 50-51 | 7 | | 4 | | | 1 | | | | | 12 |
| 1805 m | 75-76 | 6 | 3 | 3 | | | | | | | | 12 |
| | 90-91 | 12 | | | | | | | | | | 12 |
| | 120-121 | | | | | | | | | | | |
| | 130-131 | | | | | | | | | | | |
| BO03-PC03 | 11-12 | | | | | | | | | | | |
| 34°10.03'N | 17-18 | | | | 1 | 9 | 2 | | | | | 12 |
| 137°59.05'E | 35-36 | 5 | 5 | | | | | | | | 1 | 11 |
| 855 m | 52-53 | 8 | 3 | | | | | | | | | 11 |
| | 60-61 | | | | | | | | | | | |
| | 71-72 | | | | | | | | | | | |
| BO03-PC05 | 13-14 | 8 | 3 | | 1 | | | | | | | 12 |
| 34°14.06'N | 25-26 | 8 | 4 | | | | | | | | | 12 |
| 137°40.25'E | 35-36 | 12 | | | | | | | | | | 12 |
| 1186 m | 45-46 | 12 | | | | | | | | | | 12 |
| | 55-56 | 11 | | 1 | | | | | | | | 12 |
| | 64-65 | | | | | | | | | | | |
| BO03-PC06 | 7-8 | | 10 | | 2 | | | | | | | 12 |
| 34°12.33'N | 23-24 | | | | | | | | | | | |
| 137°27.56'E | 44-45 | 7 | 5 | | | | | | | | | 12 |
| 1270 m | 74-75 | | | | | | | | | | | |
| | 94-95 | | | | | | | | | | | |
| | 115-116 | | | | | | | | | | | |
| | 145-146 | | | | | | | | | | | |
| | 160-161 | | | | | | | | | | | |
| | 175-176 | | | | | | | | | | | |
| | 195-196 | | | | | | | | | | | |
| | 215-216 | | | | | | | | | | | |
| BO03-PC07 | 25-26 | | | | | | | | | | | |
| 34°10.57'N | 44-41 | | | | | | | | | | | |
| 137°25.34'E | 60-61 | | | | | | | | | | | |
| 1208 m | 80-81 | | | | | | | | | | | |
| | 97-98 | | | | | | | | | | | |

Table 1: Continued

| Core code Latitude Longitude Water depth | Section (cm below seafloor) | Operational *amoA*(β) unit (OAU) [Accession number AB261611-AB261620] 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | Sectional clone number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| BO04-PC02 | 15-17 | 8 | | | | | 3 | | | | | 11 |
| 33°54.89'N | 47-49 | 12 | | | | | | | | | | 12 |
| 137°14.01'E | 87-89 | | | | | | | | | | | |
| 1591 m | 107-109 | | | | | | | | | | | |
| | 152-154 | | | | | | | | | | | |
| | 202-204 | | | | | | | | | | | |
| | 252-254 | | | | | | | | | | | |
| | 302-304 | | | | | | | | | | | |
| | 331-333 | | | | | | | | | | | |
| BO04-PC03 | 23-28 | 12 | | | | | | | | | | 12 |
| 33°49.16'N | 46-48 | 10 | | 1 | | | | | | | | 11 |
| 136°30.06'E | 67-69 | 8 | | 3 | | | | | | | | 11 |
| 2053 m | 90-92 * | 12 | | | | | | | | | | 12 |
| | 115-117 | 3 | | | 9 | | | | | | | 12 |
| | 140-142 | 5 | 6 | | | | | | | | | 11 |
| | 187-189 | | | | | | | | | | | |
| | 237-239 | 12 | | | | | | | | | | 12 |
| | 287-289 | 12 | | | | | | | | | | 12 |
| | 337-339 | 12 | | | | | | | | | | 12 |
| | 387-389 | | | | 11 | | | | | | | 11 |
| | 437-439 | | | | | | | | | | | |
| BO04-PC05 | 12-14 | | | | | | | | | | | |
| 33°57.66'N | 37-39 | | | | | | | | | | | |
| 137°17.27'E | 62-64 | | | | | | | | | | | |
| 1314 m | 87-89 | | | | | | | | | | | |
| | 112-114 | | | | | | | | | | | |
| | 137-139 | | | | | | | | | | | |
| | 152-154 | | | | | | | | | | | |
| BO04-PC08 | 12-14 | | | | | | | | | | | |
| 34°10.61'N | 37-39 | | | | | | | | | | | |
| 137°25.31'E | 67-69 | | | | | | | | | | | |
| 1206 m | 87-89 | | | | | | | | | | | |
| | 112-114 | | | | | | | | | | | |
| | 143-145 | | | | | | | | | | | |
| OAU clone number | | 293 | 45 | 28 | 28 | 9 | 7 | 1 | 1 | 1 | 1 | Total 414 clones |

*The OPU1 and OPU2 were retrieved from 90-92 cm in BO04-PC03 and 20-21 cm in BO03-PC02, respectively

### DNA Extraction and PCR Amplification

Bulk DNAs were extracted from 200 mg each of thawed samples by bead-beating (Miller *et al*., 1999) with 2 g each of 0.1 mm zirconium silica beads in 0.3 mL of phosphate buffer (100 mM $NaH_2PO_4$; pH 8.0), 0.3 mL of lysis buffer (10% SDS, 100 mM NaCl and 500 mM Tris; pH 8.0) and 0.3 mL of chloroform-isoamyl alcohol (24: 1) in 2 mL tubes. A Mini Bead Beater-8 (Biocompare, Inc., South San Francisco, Calif.) was used at 1100 x g for 4 min to liberate DNA and 0.1 mL each of supernatants after centrifugation at 21600 x g for 5 min was filter-purified with a Chroma Spin+TE1000 Column (BD Biosciences, San Jose, USA). The bulk DNAs in the filtrates were amplified using the GenomiPhi DNA Amplification Kit (GE Healthcare, Buckinhamshire, UK) and then used as PCR templates for *amoA*, *pmoA* and *mmoX*.

The amplified bulk DNAs were purified using the QIAquick PCR Purification Kit (Qiagen Inc., Valencia, Calif.) and prepared at 70 ng $\mu L^{-1}$ each, which were tested by PCR with the primers for bacterial 16S rRNA gene (Lane, 1991). Then the bulk DNAs were used to amplify the *amoA*(β), *amoA*(γ), *pmoA* and *mmoX* sequences using ExTaq DNA polymerase (TaKaRa Bio Inc., Otsu, Japan)

Table 2: PCR primer sets and conditions for amplification of the *amoA*(β), *amoA*(γ), *pmoA* and *mmoX* sequences. K = T or G; M = C or A; N = A, T, C or G; S = G or C; Y = C or T

| Target gene (References) | PCR forward (F) and reverse (R) primer set | PCR condition Denaturation | Annealing | Extension | Cycles |
|---|---|---|---|---|---|
| *amoA*(β) Rotthauwe *et al*., 1997 (Purkhold *et al*., 2000) | F5'-GGGGTTTCTACTGGTGGT-3' R5'-CCCCTCKGSAAAGCCTTCTTC-3' | 94°C, 15 sec | 55°C, 20 sec | 72°C, 40 sec | 30 |
| *amoA* (γ) (Purkhold *et al*., 2000) | F5'-GGTGAGTGGGYTAACMG-3 R5'-GCTAGCCACTTTCTGG-3' | 94°C, 15 sec | 48°C, 20 sec | 72°C, 40 sec | 30 |
| *pmoA* (Costello and Lidstrom, 1999) | F5'-GGNGACTGGGACTTCTGG-3' R5'-CCGGMGCAACGTCYTTACC-3' | 92°C, 60 sec | 55°C, 90 sec | 72°C, 60 sec | 25 |
| *mmoX* (Miguez *et al*., 1997) | F5'-CGGTCCGCTGTGGAAGGGC ATGAAGCGCGT-3' R5'-GGCTCGACCTTGAACTTGG AGCCATACTCG-3' | 94°C, 60 sec | 55°C, 60 sec | 72°C, 60 sec | 30 |

with a TaKaRa Cycler 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) shown in Table 2.

**Cloning, Sequencing and 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.). Twelve transformants per sample, if PCR-positive, were randomly collected and sequenced bi-directionally by the dideoxy method (Sanger *et al*., 1977) on a 3730×l 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 AB261611 to AB261622. 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 the libraries. Ammonia monooxygenase and methane monooxygenase are membrane-bound (Hanson and Hanson, 1996). Transmembrane-spanning regions and topology of the deduced proteins were estimated using the TMHMM tools (br.expasy.org/tools/).

## RESULTS AND DISCUSSION

The *amoA*(β) and *pmoA* sequences were retrieved from 37 and 2 sections, respectively, out of total 79 (Table 1). The chimera-checked clones that have > 97% nucleotide similarities were grouped into operational *amoA*(β) and *pmoA* units (ten OAUs and two OPUs) from 414 and 15 clones, respectively. No *amoA*(γ) and *mmoX* sequences were amplified despite repeated PCR trials with the standard (Table 2) and modified thermal cycles.

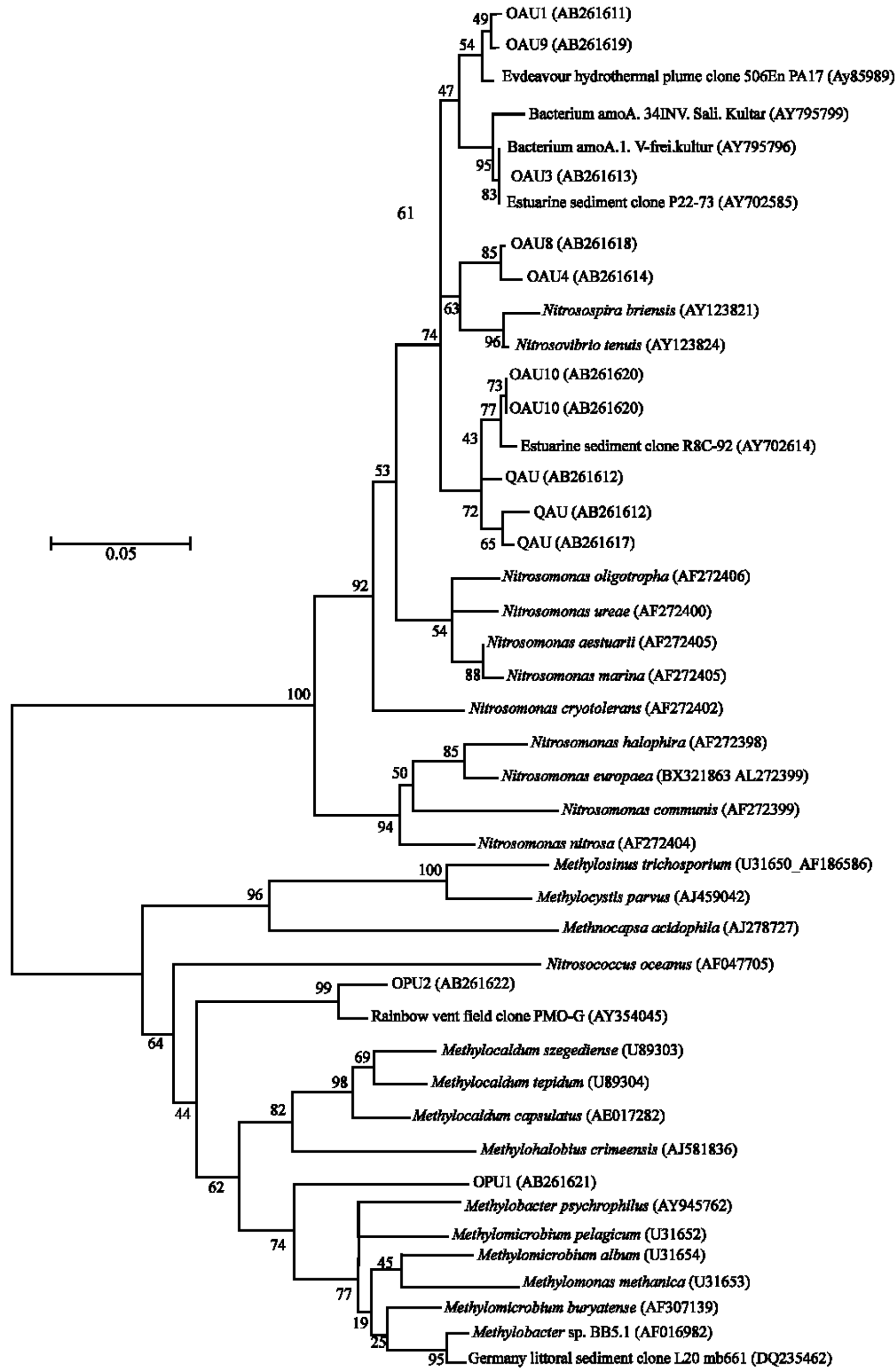


Fig. 1: Phylogenetic tree based on inferred 102 amino acids of *amoA*(β) and *pmoA* (OAUs and OPUs, respectively) from the Nankai Trough deep-sea sediment cores

Table 3: Numbers of amino acid residues, numbers of transmembrane (TM) regions and TM amino acid positions inferred from the environmental *amoA*(β) and *pmoA* clones and from *Nitrosomonas europaea* and *Methylococcus capsulatus*

| Target gene | Operational units/ known species | Amino acid residues regions | TM | TM amino acid positions |
|---|---|---|---|---|
| *amoA*(β) | OAU 1, 2, 4, 7-10 | 163 | 3 | 10-32, 37-59, 108-130 |
| | OAU 3, 6 | 163 | 3 | 10-32, 37-59, 107-129 |
| | *N. europaea* | 163 (full 276) | 3 | 10-32, 37-59, 108-130 |
| *pmoA* | OPU 1, 2 | 169 | 4 | 13-35, 40-57, 64-86, 90-112 |
| | *M. capsulatus* | 169 (full 247) | 4 | 13-35, 40-57, 64-86, 90-112 |

The *amoA*(β) and *pmoA* sequences encoded transmembrane regions of the corresponding enzymes AMO and pMMO, respectively and the numbers and lengths of putative transmembrane regions were compared with known counterparts of *Nitrosomonas europaea* and *Methylococcus capsulatus* (Table 3). The phylogenetic tree of deduced amoA and pmoA amino acid sequences (Fig. 1) shows the phylogenetic relatedness and diversity of the genes.

The OAU1 predominated the *amoA*(β) population with 293 clones (70.8% of total), while all the OAUs were inter-related at >84.7% nucleotide similarities and commonly related to the environmental *amoA*(β) clone from an inland salt habitat (AY795799) at nucleotide similarities of 82.7% (OAU10) to 95.1% (OAU3). In contrast, inferred amino acid sequences suggested that OAU1, 4, 5 and 7-10 were most closely related to the environmental *amoA*(β) clone (AY785989) from the Endeavour hydrothermal plume at 95.7-99.4% similarities. OAU2, 3 and 6 were closely related to the environmental *amoA*(β) clones from estuarine sediment (AY702585 and AY702614; Bernhard *et al*., 2005) at 96.9-98.8% similarities.

The OPU1 was major in the *pmoA* population with 11 clones (73.3% of total) and most closely related to *pmoA* of the estuarine methanotroph, *Methylobacter* sp. BB5.1 (AF016982) based on nucleotides and to the environmental *pmoA* clone from littoral sediment (DQ235462; Bussmann *et al*., 2006) based on amino acids, at 81.5 and 87.6% similarities, respectively. On the other hand, OPU2 was most closely related to *pmoA* of thermophilic *Methylocaldum szegediense* (U89303; Bodrossy *et al*., 1997) based on nucleotides and to the environmental *pmoA* clone from a hydrothermal vent field (AY354045) based on amino acids, at 75.8 and 97.6% similarities, respectively.

Heterogeneity, or patchiness, was the key distribution pattern of *amoA*(β) and *pmoA* in the studied sediment cores/sections (Table 1). Detailed geological and geochemical data will be reported elsewhere. Ambient pH and salinity may not explain the patchiness, although these factors influence distributions of ammonia-oxidizing bacteria (Bernhard *et al*., 2005; Pommerening and Koops, 2005). This study conducted a basin-wide survey is to collect diverse and novel *amoA/pmoA* against the patchiness, while patchy localization of specific *amoA/pmoA* is currently unpredicted by observed sediment features such as porosity, grain size (clay, silt to sand) and interstitial sulfate concentration.

## CONCLUSIONS

This study displayed a catalog of *amoA*(β) and *pmoA*, the genes coding for enzymes probably involved in ammonia oxidation in deep sea sediments. In future study, using RNA instead of DNA may allow us to determine whether these gene sequences are actually from active cells or not.

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