Distinctive physiological response of shallow and deep ecotypes of ammonia-oxidizing marine archaea in seawater cultures

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Abstract: Ammonia-oxidizing marine archaea (mAOA) have been divided into two groups based on ammonia mono-oxygenase subunit A gene (amoA) phylogeny and they link to preferred habitat (depth in the water column); one is the Shallow Marine Clade (SMC) and the other is the Deep Marine Clade (DMC). Several determinative factors have been proposed to account for their depth-dependent distributions, including light intensity, ammonia concentration, water temperature, and dissolved oxygen concentration. Here, we report the change in abundance of SMC and DMC mAOA in response to nitrogen source and water temperature in a long-term incubation experiment using natural seawaters obtained from mesopelagic and bathypelagic layers. The abundance of each type of mAOA was determined by Q-PCR assay targeting amoA. We found that the abundance of SMC after 266 days of incubation increased at all three incubation temperatures (4, 10, 20°C), whereas the abundance of DMC was stable at low temperatures (4° C and sometimes 10°C) or decreased to below the detection limit at high temperatures (20° C and sometimes 10°C). The SMC abundance was higher in seawater cultures supplied with ammonium than in those supplied with nitrite, although DMC abundance was not responsive to ammonium concentration. These results imply that water temperature and ammonia concentration are significant factors in determining the vertical distribution of the SMC and DMC ecotypes of mAOA in the water column.

Key words: Ammonia-oxidizing archaea, Temperature, Ammonia, Growth factor, Suruga Bay

Introduction

Marine Group I (MGI) is one of the major marine archaeal clades in the phylum Thaumarchaeota (Brochier-Armanet et al. 2008, Delong 1992, Fuhrman et al. 1992). Some of the marine archaea belonging to MGI are able to oxidize ammonia (Könneke et al. 2005, Muller et al. 2010 and so on.). These ammonia-oxidizing marine archaea (mAOA) are divided into two groups depending on their phylogeny with respect to the ammonia monooxygenase subunit A gene (amoA) and they link to preferential depth of distribution (Beman et al. 2008, Mincer et al. 2007, Santoro et al. 2010). One group is called the Shallow Marine Clade (SMC) and the other is the Deep Marine Clade (DMC). In some studies, the mAOA has been divided into three groups, the Nitrospumilus maritimus-like Cluster (NMC), Water Column Cluster A (WCA), and Water Column Cluster B (WCB) (Beman et al. 2008, Francis et al. 2005, Hallam et al. 2006). NMC and WCA are subdivisions of SMC, and WCB corresponds with DMC in the AmoA phylogeny (Hallam et al. 2006).

Studies of the environmental factors determining the distribution of each cluster of mAOA were reviewed in order to understand the ecology and physiology of mAOA (Erguder et al. 2009). Although the important factors determining their distribution have been reported to include sunlight, water temperature, ammonia concentration, and dissolved oxygen concentration in the marine water column (Hallam et al. 2006, Mincer et al. 2007, Molina et al. 2010), the primary determinants remain unresolved. Candidatus Nitrosopumilus maritimus SCM1, the first isolate of mAOA, was indicated to have a higher affinity for ammonium than phytoplankton and bacteria, even those known to be ammonia-oxidizers (Martens-Habbena et al. 2009). This feature was seen as an adaptation to marine environments that tend to be nitrogen limited. In a study by Molina et al. (2010), the proportion of the archaea popu-
lotion belonging to WCA was positively correlated with ammonium and dissolved oxygen concentrations, indicating the influence of these environmental factors on the abundance of this group, whereas the proportion belonging to WCB was not correlated with these factors, suggesting that other environmental factors are more important for this group. Also, our previous study showed that an increase in water temperature from 4 to 20°C drastically changed the proportions of DMC and SMC in seawater cultures, suggesting the importance of temperature in determining their relative distributions in the water column (Ijichi & Hamasaki 2011).

In this study, in order to test the effect of temperature as a factor in determining SMC and DMC distributions, we further analyzed the seawater cultures established in our previous studies and examined the difference between SMC and DMC, especially pertaining to abundance, in response to temperature increase in seawater cultures. We also examined the effect of inorganic nitrogen (ammonia) supply on each ecotype.

Materials and Methods

Sample collection and seawater cultures

Water samples were collected from depths of 500 m and 2000 m in Suruga Bay, Japan (34°38’N, 138°03’E; maximum depth, 2330 m) during cruise KT-08-02 of R/V Tansei-maru in February 2008, and 12 seawater cultures were prepared; six from each depth. Water temperatures were measured with a CTD profiler (ICTD; Falmouth Scientific, Inc., Cataumet, Massachusetts, USA) and DO concentrations with a DO sensor (Beckman polarographic oxygen sensor; Falmouth Scientific, Inc.). Concentrations of inorganic nitrogen were measured with a segmented continuous flow analyzer (swAAAt; BLTEC, Tokyo, Japan). Details of nutrient measurements were previously described by Ijichi & Hamasaki (2011). Seawater temperatures were 6.6°C at 500 m and 2.0°C at 2000 m. At these two depths, DO concentrations were 126 µM and 150 µM, ammonium concentrations were 0.31 µM and 0.91 µM, nitrite concentrations were <0.05 µM and 0.34 µM, and nitrate concentrations were 31.3 µM and 39.1 µM, respectively. In total, 12 seawater cultures were maintained for up to 1200 days in darkness without shaking. The initial volume of seawater per culture was 500 mL. Culture vessels were 1-L polypropylene bottles with airtight screw caps. Half of them received sample water from 500-m depth, and the other half received water from 2000-m depth. Three of the six cultures from each depth were supplied with (NH₄)₂SO₄ and the other three were supplied with NaNO₂ at the onset of incubation (50 µM final concentration of NH₄⁺ or NO₂⁻) and once or twice thereafter. Each of the three cultures in each set were incubated at a different temperature: 4, 10, and 20°C, respectively (n=1). This meant that four different seawater cultures (500-m SW+NH₄⁺, 500-m SW+NO₂⁻, 2000-m SW+NH₄⁺, 2000-m SW+NO₂⁻) were incubated at each of three temperatures. The NaNO₂ treatment was a control for comparison with the cultures supplied with (NH₄)₂SO₄, because nitrite production was expected after the addition of (NH₄)₂SO₄. The timing of (NH₄)₂SO₄ and NaNO₂ addition was determined by monitoring the decrease or increase in inorganic nitrogen concentration in the cultures. Concentrations of inorganic nitrogen were intermittently measured with 10-mL subsamples using a segmented continuous flow analyzer (swAAAt; BLTEC) as described previously (Ijichi & Hamasaki 2011). After 266, 915, 978 and 1200 days of incubation, 30- to 50-mL subsamples of seawater for DNA extraction were taken from each bottle, centrifuged (15,300×g, 30 min, 4°C), and then frozen at −20°C until further analysis. Nucleic acids were extracted from centrifuged pellets using ChargeSwitch Forensic DNA Purification Kits (Invitrogen, Carlsbad, California, USA) following the manufacturer’s instructions. DNA concentrations were measured with Quant-it PicoGreen dsDNA Reagent and Kits (Invitrogen) following the manufacturer’s instructions. Culture names, sampling information, culture conditions, and references are listed in Table 1.

Quantitative polymerase chain reaction analyses

Q-PCR assays for archaeal amoA were performed on a LightCycler 480 Real-Time PCR System (Roche Applied Science, Mannheim, Germany). Three different primer sets for general AOA, SMC, and DMC were used (Francis et al., 2005 and Beman et al., 2008). The primer set for general AOA is the universal archaeal amoA primer set targeting all AOA. The SMC primer set specifically targets amoA of NMC and WCA. The DMC primer set specifically targets amoA of WCB. The reaction mixtures (20 µL) contained 10 µL of SYBR Premix Ex Taq (TaKaRa, Shiga, Japan), 0.2 µM of each primer, and 2 µL of DNA. Assays were run in triplicate under the following cycling condi-

| Seawater culture name | Collected depth (m) | Culture temperature (°C) | Nitrogen source | Reference |
|----------------------|---------------------|--------------------------|-----------------|-----------|
| KT-122               | 500                 | 4                        | (NH₄)₂SO₄       | Ijichi and Hamasaki (2011) |
| KT-124               | 2000                | 10                       |                 |           |
| KT-126               | 500                 | 10                       |                 |           |
| KT-128               | 2000                | 20                       |                 |           |
| KT-130               | 500                 | 4                        | NaNO₂           | This study |
| KT-132               | 2000                | 10                       |                 |           |
| KT-134               | 500                 | 20                       |                 |           |
| KT-136               | 2000                | 20                       |                 |           |
| KT-138               | 500                 | 4                        |                 |           |
| KT-140               | 2000                | 10                       |                 |           |
| KT-142               | 500                 | 20                       |                 |           |
| KT-144               | 2000                | 4                        |                 |           |
tions: 95°C for 30 sec, followed by 45 cycles of 95°C for 15 sec; 53°C (general AOA), 56°C (SMC) or 55°C (DMC) for 30 sec; and 72°C for 30 sec; with a detection step at the end of each cycle. Gene copy numbers per unit volume of sub-sample (or seawater sample) was calculated based on DNA concentration of extracts and its amount used for Q-PCR. Primer information, standard information, standard curve correlation coefficients, and PCR efficiencies are listed in Table S1.

**Sequencing and phylogenetic analysis of the amoA**

Since we found a significant increase in SMC abundance in all seawater cultures after 266 days, amoA fragments were amplified to determine detailed phylogenotypes of SMC mAOA found in this study. Two seawater cultures, named KT-130 (500-m SW + NH₄⁺ incubated at 20°C) and KT-1132 (2000-m SW + NH₄⁺ incubated at 20°C), were used for this examination (Table 1). We used a previously described primer set, Arch-amoAFA (Beman et al. 2008) and Arch-amoaAR (Francis et al. 2005), for SMC-specific amoA amplification. Two other primer sets, amoA-1F and amoA-2R for betaproteobacterial amoA (Rotthauwe et al. 1997), and amoA-3F and amoA-4R for gammaproteobacterial amoA (Purkhold et al. 2000) were also used, but these primer sets gave no PCR products from any samples tested in this study. Amplified fragments were cloned by using a TOPO TA Cloning Kit for Sequencing (Invitrogen) and *E. coli* DH5α Competent Cells (TaKaRa), and sequenced with a BigDye v.3.1 Sequencing Kit (Applied Biosystems, Foster City, California, USA). Clones with ≥98% nucleotide sequence identity were assigned to the same representative type by using the CD-HIT suite with default settings (Huang et al. 2010). Nucleotide sequences were aligned with MUSCLE software within the MEGA5 package (Tamura et al. 2011). For assigning OTUs in this study, representative sequences were compared with previously reported sequences from environmental clones, marine metagenomic libraries, enrichment cultures, isolated strains and single cell genomes. Phylogenetic trees were inferred by the neighbor-joining method (Saitou & Nei 1987), the evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2011). The nucleotide sequences were submitted to the DNA Databank of Japan (DDBJ) and were assigned the following accession numbers: AB919450 to AB919587 for amoA.

**Results**

**Inorganic nitrogen in seawater cultures**

Nitrite and/or nitrate concentrations increased with decreasing ammonium concentrations in all cultures supplied with (NH₄)₂SO₄ (Fig. 1a–f, modified from Ijichi & Hamasaki 2011). Nitrate concentrations increased with decreasing nitrite concentration in all seawater cultures supplied with NaNO₂, except the 2000-m seawater cultures at 4°C (Fig. 1g–i).

**Selection of primer sets for mAOA abundance**

The total amoA copy number (total AOA), calculated as the sum of the SMC and DMC copy numbers, was unexpectedly higher than that determined by using the general AOA primer set in almost all samples containing positively detected AOA. The ratio of the total AOA count to the general AOA count for environmental samples was 1.47 for 500-m and 1.76 for 2000-m seawater and ranged from 0.51 to 558 for the incubated seawater culture samples (n = 34; Fig. S1). Therefore, we used the total AOA number as a measure of the abundance of total mAOA.

**Abundance of SMC and DMC**

In all seawater cultures after 266 days of incubation, the abundance of SMC increased by 1–3 orders of magnitude (Fig. 2a and b), and was significantly higher (Student's t-test, *P*<0.05) in cultures supplied with (NH₄)₂SO₄ than in those with NaNO₂ (Fig. S2). The increased abundance of SMC persisted to 1200 days in the cultures at 4°C (Fig. S2a and d). However, it decreased at the higher temperatures of 10°C and 20°C, especially in the cultures supplied with (NH₄)₂SO₄ (Fig. S2b, c, e and f).

In contrast, the abundance of DMC did not show a significant increase in any of the cultures during the first 266 days of incubation and even decreased or disappeared thereafter (Fig. 2c and d, Fig. S3). The reduction in abundance became more pronounced as temperature increased (Fig. 2c and d).

Although we didn’t analyze exact replicates for each condition, cultures from 500-m and 2000-m samples showed similar trends and could be a replicate of temperature and ammonium responses of AOA. For example, the trend of SMC increase at all three temperatures and DMC decrease at high temperatures was similarly observed in both 500-m and 2000-m cultures. Also, the higher SMC abundance in ammonium amendment cultures was similarly observed in both 500-m and 2000-m cultures.

**Phylogenotypes of SMC**

The AOA community in the KT-130 (500-m SW + NH₄⁺ incubated at 20°C) culture at the onset of incubation was represented by 12 operational taxonomic units (OTUs) belonging to NMC (comprising 80.9% of total clones), 6 OTUs belonging to WCA (17.0%), and 1 OTU belonging to Water Column Cluster A2 (WCA2; 2.1%), whereas at day 266 it was represented by 4 OTUs belonging to NMC (77.3%) and 1 OTU belonging to WCA (22.7%) (Fig. 3a and c, Fig. S4, Table S2). WCA2 is one of the subclusters in the Water Column Cluster containing WCA and WCB (Moraru et al. 2010).
The AOA community of the KT-132 (2000-m SW $+\text{NH}_4^+$) incubated at 20°C) culture at the onset of incubation was represented by 11 OTUs belonging to NMC (78.7%), 7 OTUs belonging to Sediment Cluster A-1 (SCA-1; 17.0%) and 2 OTUs belonging to WCA (4.3%); at day 266 it was represented by 2 OTUs belonging to NMC (100%) (Fig. 3b and d, Fig. S4, Table S2). SCA-1 is one of the subclusters in the Sediment Cluster containing NMC (Nakagawa et al. 2007).

Discussion

Previous studies have reported that SMC was predominant in aquaria and shallow or coastal seas, whereas DMC was predominant in the deep sea (Beman et al. 2008, Hu et al. 2011a,b, Santoro et al. 2010, Urakawa et al. 2008). The present study, an enrichment culture experiment using natural seawater, suggested contrasting features of the two ecotypes of marine AOA in regards to temperature response. Incubation at higher temperature (10°C and 20°C) than the ambient temperatures of the sampling depths caused a drastic decrease in DMC abundance, suggesting their vulnerability to higher temperatures. Since their abundance at 4°C remained constant, DMC cells might be under extremely slow growth or a dormant state. In either

Fig. 1. Inorganic nitrogen concentrations during 300-day incubations of seawater samples in two major treatment groups: (1) supplied with (NH$_4$)$_2$SO$_4$ and collected from 500-m depth and incubated at (a) 4°C, (b) 10°C, and (c) 20°C or collected from 2000 m and incubated at (d) 4°C, (e) 10°C and (f) 20°C, or (2) supplied with NaNO$_2$ and collected from 500-m depth and incubated at (g) 4°C, (h) 10°C, and (i) 20°C or collected from 2000 m and incubated at (j) 4°C, (k) 10°C, and (l) 20°C ($n=1$). Panels a–f are modified from Fig. 2 of Ijichji and Hamasaki (2011). NH$_4^+$-N (blue diamonds); NO$_2^-$-N (red squares); NO$_3^-$-N (green triangles); Addition of substrate (black triangles). In h, k, i and l, some blue diamonds were behind the red squares, and double red squares and double green triangles indicate before and after substrate supply, respectively.

Fig. 2. Ratios of amoA copy number at day 266 of incubation to those at day 0 in the seawater cultures. The data show the Shallow Marine clade from the seawater cultures from a depth of about (a) 500 m and (b) 2000 m, and the Deep Marine clade from the seawater cultures from a depth of about (c) 500 m and (d) 2000 m. Bars indicate the seawater cultures supplied with (NH$_4$)$_2$SO$_4$ (white) and NaNO$_2$ (black). ND (not detected) indicates below the detection limit and their bars indicate the value of the detection limit. NT (not tested) indicates absent sample.

The AOA community of the KT-132 (2000-m SW $+\text{NH}_4^+$) incubated at 20°C) culture at the onset of incubation was represented by 11 OTUs belonging to NMC (78.7%), 7 OTUs belonging to Sediment Cluster A-1 (SCA-1; 17.0%) and 2 OTUs belonging to WCA (4.3%); at day 266 it was represented by 2 OTUs belonging to NMC (100%) (Fig. 3b and d, Fig. S4, Table S2). SCA-1 is one of the subclusters in the Sediment Cluster containing NMC (Nakagawa et al. 2007).
The abundance of SMC was higher in seawater cultures supplied with (NH$_4$)$_2$SO$_4$ than in those with NaNO$_2$ after 4–10°C. In contrast, SMC abundance increased at the higher incubation temperatures, suggesting an eurythermal physiology. Our previous study showed a shift in community structure from DMC dominance to SMC dominance after incubation at higher temperature (10°C and 20°C) (Ijichi & Hamasaki 2011). The current study suggests that this shift in community structure was caused by the increase of SMC cells and decrease of DMC cells at higher temperatures. Sunlight, ammonium concentration, and DO concentration have all been proposed as factors determining the distinctive vertical distribution of SMC and DMC ecotypes (Hallam et al. 2006, Mincer et al. 2007, Molina et al. 2010). Our previous and current studies provide strong evidence that temperature is another important factor. The habitable area for DMC or WCB is possibly limited to depths or regions where water temperature never rises much above 4–10°C.

The abundance of SMC was higher in seawater cultures supplied with (NH$_4$)$_2$SO$_4$ than in those with NaNO$_2$ after 266 days of incubation, suggesting that ammonium concentration controls SMC abundance. SMC also grew in the cultures to which NaNO$_2$ was added. They might use a trace amount of ammonium contained in natural seawater (presumably <10 nM), which is undetectable with the current conventional analytical method. For example, Can-

\[\text{N. maritimus SCM1 belonging to SMC, grew exponentially with depleted ammonium concentration below 10 nM. This ammonium concentration approaches values measured in the oligotrophic open ocean (Martens-Habbena et al. 2009). A previous study suggested the presence of two mAOA ecotypes, one adapted to the ammonium concentrations associated with shallow water and one to those of deep waters (Sintes et al. 2013). The HAC-AOA ecotype (high ammonia concentration AOA) was dominant in coastal Arctic waters and the surface waters of the open equatorial Atlantic, whereas the LAC-AOA ecotype (low ammonia concentration AOA) was dominant in the meso- and bathypelagic waters of the tropical Atlantic, where ammonium concentrations are below the detection limit. The HAC-AOA and LAC-AOA ecotypes approximately corresponded to SMC and DMC, respectively. Our results, showing a long-term positive effect of elevated ammonium concentration on the abundance of SMC, support the idea of ecotype determination by affinity for ammonia utilization. However, it still remains to be determined whether DMC is adapted to lower ammonia concentrations than is SMC.}

In regard to the phylotypes of SMC, Beman et al. (2008) reported that sequences unique to WCA were recovered from all of their clone libraries obtained from water columns of the Guaymas Basin and the Carmen Basin in the Gulf of California. Also, WCA has been frequently recovered in marine metagenomic libraries obtained from the Sargasso Sea; from an oceanographic cruise from Newport, Oregon, to Honolulu, Hawaii; from Station ALOHA; and from Monterey Bay, California (Venter et al. 2004, Hallam et al. 2006, Mincer et al. 2007). However, NMC sequences were the most frequently recovered from marine metagenomic libraries obtained from the Gulf of Maine (Tully et al. 2012). We also found that the phylotypes belonging to NMC were dominant in SMC at both the onset and at day 266 of the incubations. Such a discrepancy in the dominant phylotype of SMC may be due to the different geographical distributions of NMC and WCA. WCA is abundant on the eastern side of oceanic basins adjacent to the western coasts of continents, whereas NMC is abundant on the western side of oceanic basins adjacent to the eastern coasts of continents. Alternatively, it may be due to a difference in methodology. The dominance of WCA by Beman et al. (2008) was obtained from sequence data derived from PCR products using the general AOA primer set. In the current study, the total amoA copy number (total AOA), calculated as the sum of the SMC and DMC copy numbers, was higher than that determined by using the general AOA primer set, which means that the general AOA primer set could miss some phylotypes because of primer mismatch, and this could lead to an underestimation of AOA abundance. This is why we used the SMC-specific primer set for SMC phylotype analysis. For further study, an alternative “general AOA” primer set should be designed to cover a broader range of mAOA diversity than

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**Fig. 3.** Proportion of various archaeal amoA sequences represented as clusters (outer circles) and OTUs (inner circles). The Shallow Marine clade primer set was used for PCR amplification of amoA. The data are shown for the seawater cultures of (a) KT-130 and (b) KT-132 at the onset of incubation (day 0), and (c) KT-130 and (d) KT-132 at day 266 of incubation. The outer circles indicate proportions of the Nitrosopumilus maritimus-like cluster (white), Sediment cluster A-1 (light gray), Water Column A cluster (black), and Water Column A2 cluster (dark gray). The inner circles indicate proportions of individual OTUs.
that which we are currently using.

In conclusion, water temperature and ammonia concentration are significant factors controlling the abundance of SMC and DMC ecotypes of mAOA, which explains their depth-dependent distribution in the water column. Factors controlling the different geographical distributions of NMC and WCA are currently unknown. Further studies should be aimed at revealing their global distribution patterns and the controlling factors.

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