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MICROBES AND ENVIRONMENTS

Enrichment Using an Up-flow Column Reactor and Community Structure of Marine Anammox Bacteria from Coastal Sediment

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We established an enrichment culture of marine anaerobic ammonium oxidation (anammox) bacteria using an up-flow column reactor fed with artificial sea water supplemented with nitrogen and minerals and inoculated with coastal surface sediment collected from Hiroshima Bay. After 2 months of reactor operation, simultaneous removal of NH_4^+ and NO_2^- was observed, suggesting that an anammox reaction was proceeding. A total nitrogen removal rate of 2.17 g-N L^{-1} day^{-1} was attained on day 594 while the nitrogen loading rate was 3.33 g-N L^{-1} day^{-1}. Phylogenetic analysis revealed that at least two dominant "*Candidatus* Scalindua" species were present in this reactor. Moreover, many uncultured bacteria and archaea, including candidate division or ammonia-oxidizing archaea, were present. Fluorescence *in situ* hybridization (FISH) revealed that anammox bacteria accounted for 85.5 ± 4.5% of the total bacteria at day 393. We also designed two oligonucleotide probes specific to each dominant "*Candidatus* Scalindua" species. A simultaneous FISH analysis using both probes showed that two different "*Candidatus* Scalindua" species were clearly recognizable and coexisted during reactor operation, although there was some variation in their abundance. The marine anammox bacteria enriched in this study have potential applications to the treatment of industrial wastewater containing high levels of ammonium and salt.

Key words: anaerobic ammonium oxidation (anammox), marine anammox bacteria, "*Candidatus* Scalindua" species, enrichment culture, FISH, phylogenetic analysis

Anaerobic ammonium oxidation (anammox) is a biological process in which ammonium is directly converted to dinitrogen gas with nitrite as the electron acceptor under anoxic conditions (9). Anammox is mediated by a group of deep-branching *Planctomycetes*-like bacteria (31). The anammox process is an efficient and cost-effective alternative to conventional processes used to remove nitrogen from ammonia-rich wastewater (9). The advantages of the anammox process over the conventional combination of nitrification and denitrification processes include lower oxygen demand and no requirement for external carbon sources. Because some types of industrial wastewater, such as landfill leachate, contain high salt concentrations, salinity is an important parameter for wastewater treatment (11). Anammox bacteria have been detected in wastewater treatment facilities and natural environments worldwide (26). Currently, at least five genera of anammox bacteria have been reported and proposed, including "*Candidatus* Brocadia", "*Candidatus* Kuenenia", "*Candidatus* Scalindua", "*Candidatus* Anammoxoglobus", and "*Candidatus* Jettenia".

The "*Candidatus* Scalindua" group is primarily found in marine environments such as the Black Sea (16) and the Arabian Sea, as well as along the coasts of Namibia, Chile, Peru and Japan (3, 37). It is currently estimated that anammox bacteria are responsible for at least 30–50% of nitrogen removal from marine ecosystems (6), although this value is not undisputed (36). Since neither marine anammox bacteria nor wastewater anammox bacteria have been isolated in pure cultures, enrichment of marine anammox bacteria is valuable for understanding a microbial community in association with the marine anammox reaction and *in situ* activities of marine anammox bacteria and for application to the treatment of industrial wastewater containing high levels of ammonium and salt. Although "*Candidatus* Scalindua" is dominant in marine environments, only a few "*Candidatus* Scalindua" species have been enriched from sea sediment (12, 20, 35). Furthermore, the cultures showed a low population density (67–90% of total bacteria) and/or low conversion rates (0.07–0.35 g L^{-1} day^{-1}) after long periods of enrichment.

We previously reported that anammox bacteria derived from wastewater were successfully enriched using up-flow fixed-bed column reactors, and that the anammox reaction was observed within 2–3 months after the reactor start-up (14, 33). These studies were conducted based on strategies specific for the selected inoculum and a short hydraulic retention time (HRT). The short HRT might prevent substrate limitation and the accumulation of inhibitory substances, as well as establish enrichment with low concentrations of media.

The present study was conducted to establish an enrichment culture of anammox bacteria in a marine environment with a high conversion rate and to investigate whether enriched anammox bacteria belong to the "*Candidatus*

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Scalindua" group. In addition, the types of bacteria and archaea that coexisted in the enriched culture were investigated. To achieve these objectives, an up-flow fixed-bed column reactor with a short HRT was used with coastal sediment collected from Hiroshima Bay as the inoculum. Phylogenetic analysis based on 16S rRNA genes and fluorescence *in situ* hybridization (FISH) were performed to investigate the community structure of enriched anammox bacteria together with coexisting microorganisms and to monitor long-term changes of dominant anammox bacterial populations.

Materials and Methods

Sediment sample

A surface sediment sample was collected from Hiroshima Bay (34°21.4'N, 132°30.7'E, water depth 7.4 m) in the Seto Inland Sea during July 2008 using a plastic core sampler. The bottom water temperature, pH, salinity, ammonia, nitrite, and nitrate concentrations were 19.4°C, 8.0, 32.1 psu, 17.9 μM, 2.9 μM, and 6.4 μM, respectively.

Enrichment

A surface sediment sample weighing 1 g (wet weight) was used an inoculum in a glass column reactor (diameter, 2.6 cm; length, 10.5 cm) with a nonwoven fabric sheet (Japan Vilene, Tokyo, Japan) as the biofilm carrier material. To confirm the existence of DNA derived from anammox bacteria in the sediment sample, PCR was conducted using *Planctomycetales*-specific primer sets and the DNA was electrophoresed prior to inoculation (see section *DNA extraction and PCR amplification*). The reactor volume was 56 cm^3, the surface area of the biofilm carrier was 54.6 cm^2, the temperature was maintained at 20°C, and the initial hydraulic retention time (HRT) was 0.9 h. A synthetic marine nutrient medium composed of the following was used: 35 g L^{-1} of SEALIFE (Marine Tech, Tokyo, Japan), an artificial sea salt used in this study, supplemented with $(NH_4)_2SO_4$ (24 to 88 mg L^{-1}), $NaNO_2$ (25 to 88 mg L^{-1}), $KHCO_3$ (500 mg L^{-1}), KH_2PO_4 (27 mg L^{-1}), $MgSO_4 \cdot 7H_2O$ (300 mg L^{-1}), $CaCl_2 \cdot 2H_2O$ (180 mg L^{-1}), and 1 mL of trace element solutions I and II (34). It should be noted that 35 g L^{-1} of SEALIFE contained the following: Na (9.14 g L^{-1}), Mg (1.23 g L^{-1}), Ca (0.34 g L^{-1}), K (0.35 g L^{-1}), Cl (16.89 g L^{-1}), SO_4^{2-} (2.3 g L^{-1}), Sr (0.007 g L^{-1}), Br (0.055 g L^{-1}), F (0.2 mg L^{-1}), B (0.9 mg L^{-1}), Li (0.16 mg L^{-1}), V (0.003 mg L^{-1}), Rb (0.11 mg L^{-1}), Mo (0.012 mg L^{-1}), and Ba (0.016 mg L^{-1}). The medium was flushed with N_2 gas for at least 1 h before the nutrients were added to achieve a concentration of dissolved oxygen (DO) below 0.5 mg L^{-1}. The total nitrogen loading and removal rates were calculated based on the concentrations of NH_4^+, NO_2^-, and NO_3^- as well as the HRT.

Analytical methods

The seawater quality (see above) was determined using a multiparameter water quality meter (AAQ1183, JFE Alec, Kobe, Japan). The NH_4^+ concentration was determined using Nessler's method with a UV-visible spectrophotometer (DR-2800, Hach, Loveland, CO, USA). The concentrations of NO_2^- and NO_3^- were determined using ion-exchange chromatography (HPLC 10Avp; Shimadzu, Kyoto, Japan) with a Shodex Asahipak NH2P-50 4D anion column (Showa Denko, Tokyo, Japan) and UV-VIS detector (SPD-10A, Shimadzu) after filtration of samples through 0.2-μm pore-size membranes (Advantec, Tokyo, Japan).

DNA extraction and PCR amplification

Total DNA was extracted from the anammox biofilm in the column reactor after 222 days of operation using a Fast DNA spin kit (MP Biomedicals, Irvine, CA, USA) according to the manufacturer's instructions. To construct three clone libraries, an anammox bacterial clone library, a total bacterial clone library, and an archaeal clone library, the 16S rRNA gene fragments from the isolated total DNA were amplified using a ONE Shot LA PCR MIX kit (Takara Bio, Ohtsu, Japan) and a *Planctomycetales*-specific primer set, pla46f (21) and univ1390r (38), a bacteria-specific primer set, eub338f (an equimolar mixture of complementary sequences of the EUB338, EUB338 I, EUB338 II, and EUB338 III probes (2, 5)) and univ1390r (38), or an archaea-specific primer set, arc109f and arc912r (18) (Table S1). The PCR conditions for the anammox bacteria were as follows: 4 min of initial denaturation at 94°C, followed by 30 cycles of 45 s at 94°C, 50 s at 58°C, and 3 min at 72°C. The final extension was conducted for 10 min at 72°C. The PCR conditions for the total bacteria were as follows: 5 min of initial denaturation at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 50°C, and 1.5 min at 72°C. The final extension was conducted for 5 min at 72°C. The PCR conditions for the archaea were as follows: 5 min of initial denaturation at 94°C, followed by 25 cycles of 30 s at 94°C, 30 s at 50°C, and 1 min at 72°C. The final extension was conducted for 5 min at 72°C. All PCRs were performed using a total volume of 50 μL containing 1 μg of DNA as the template. The PCR products were electrophoresed in a 1% (w/v) agarose gel.

Cloning and phylogenetic analysis

The PCR products were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and then cloned using a TOPO XL PCR Cloning Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cloned 16S rRNA genes were randomly selected from each clone library and subjected to sequencing. DNA sequencing was performed by Dragon Genomics Center (Takara Bio, Yokkaichi, Japan). The sequences were compared with sequences of the reference organisms using a BLAST search (1). Sequences with 97% or higher similarity were grouped into operational taxonomic units (OTUs) using the Similarity Matrix program from the Ribosomal Database Project (19). The 16S rRNA gene sequences from the anammox bacterial clone library (1,385 bp), total bacterial clone library (1,070 bp), and archaeal clone library (788 bp) were imported and aligned using Integrated Aligners in the ARB software (17), and alignments were refined manually. Phylogenetic trees were constructed using neighbor-joining (24) with Olsen correction model and maximum parsimony (Phylip DNAPARS) approaches using default settings in the ARB software with the greengenes236469 database (http://greengenes.lbl.gov/cgi-bin/nph-index.cgi). A bootstrap resampling analysis (7) of 1,000 replicates was performed using the ARB software to estimate the confidence of tree topologies.

Oligonucleotide probes, FISH, and cell counting

The 16S rRNA-targeted oligonucleotide probes used in this study were EUB338 (2), EUB338 II (5), and EUB338 III (5) for total bacteria, Amx368 (25), S-*-Sca-1129-a-A-18 (5'-TACCCGG CACAACCCGCT-3'), and S-*-Sca-1129-b-A-18 (5'-TACTCGG CATTACCCGAT-3') for anammox bacteria, and ARC915 (29), Cren512 (10), and EURY514 (10) for archaea (S-Table 1). Probes S-*-Sca-1129-a-A-18 and S-*-Sca-1129-b-A-18 were designed in this study using the PROBE_DESIGN tool of the ARB software (17) with the greengenes236469 database. The specificity of the probes was checked based on a comparative analysis of all sequences in the ARB database which comprised both publicly available sequences and our clone sequences. To determine the formamide concentration for optimum stringency, the designed probes were analyzed on a paraformaldehyde-fixed anammox biofilm applying hybridization buffer containing 0–60% formamide (5% increments). The probes were labeled with fluorescein isothiocyanate (FITC) or tetramethylrhodamine 5-isothiocyanate (TRITC). Dehydration and FISH were performed as described previously (23). Hybridization with the probes requiring different stringency conditions was conducted using successive procedures; hybridization with the probe requiring higher stringency was always

performed first. Either a LSM5 PASCAL confocal laser-scanning microscope equipped with an Ar ion laser (488 nm) and an HeNe laser (543 nm) (Carl Zeiss, Oberkochen, Germany), or an Axioimager M1 epifluorescence microscope (Carl Zeiss) was used for observation of the samples.

For the quantitative determination of microbial composition in the anammox biofilm, surface fractions of the specific probe-hybridized cell area and EUB338mix (composed of EUB338, EUB338 II, and EUB338 III) probe-hybridized cell area were determined following homogenization of biofilm samples and simultaneous *in situ* hybridizations with various probe sets. The average surface fraction was determined from at least 10 representative laser scanning micrographs obtained from each biofilm sample using image analysis software provided by Zeiss (13).

The total number of cells was determined by 4',6'-diamidino-2-phenylindole (DAPI) direct counting. Briefly, marine anammox biofilm samples (approximately 1.0 cm^2) were collected from the reactor, homogenized, and suspended in 1 mL of phosphate-buffered saline (PBS) (10 mM sodium phosphate buffer and 130 mM sodium chloride; pH 7.2).

Nucleotide sequence accession numbers

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of the 47 OTU sequences used for the phylogenetic tree are AB573102 to AB573148.

Results and Discussion

Enrichment of marine anammox bacteria

An up-flow fixed-bed column reactor was used for the enrichment of marine anammox bacteria in this study. Before the enrichment, we examined the existence of DNA derived from anammox bacteria in the sediment sample to select an appropriate inoculum for rapid start-up of the anammox reactor. The DNA band amplified by the *Planctomycetales*-specific primers was observed by electrophoresis in the 1% agarose gel (data not shown). Consequently, one gram of the sediment was inoculated on a nonwoven fabric sheet in the glass column reactor. Upon start-up of the enrichment, the synthetic marine nutrient medium containing 5 mg-N L^{-1} of ammonium and 5 mg-N L^{-1} of nitrite was fed to the column reactor with an HRT of 0.9 h. This feeding condition was maintained for 4 months. The simultaneous removal of NH_4^+ and NO_2^- was observed after 68 days of incubation (Fig. 1A), suggesting that an anammox reaction was occurring in the reactor. During this period, the NH_4^+ and NO_2^- removal efficiencies were 30% and 59%, respectively. A 97% NO_2^- removal efficiency was observed on day 114 (Fig. 1A). After day 114, the nitrogen loading rate gradually increased as the NH_4^+ and NO_2^- concentrations increased (Fig. 1B). It should also be noted that higher nitrogen loading rates on days 172 and 276 were due to pump failure. When the nitrogen removal rate dropped suddenly, the nitrogen loading rate decreased due to a decreasing flow rate (increasing HRT); however, the nitrogen removal rate then gradually recovered. The color of the biofilm changed from gray to khaki after about 60 days and from khaki to orange after 300 days. After 300 days of reactor operation, the nitrogen loading rate gradually increased as the HRT was reduced to 0.3 h (Fig. 1B). A total nitrogen removal rate of 2.17 g-N L^{-1} day^{-1} was attained on day 594, while the nitrogen loading rate was 3.33 g-N L^{-1} day^{-1} (HRT, 0.3 h). The average nitrogen stoichiometric ratio after 300 days of operation was 1:1.28:0.24 for consumption of NH_4^+ and NO_2^- for the production of NO_3^-. This stoichiometric ratio was similar to that

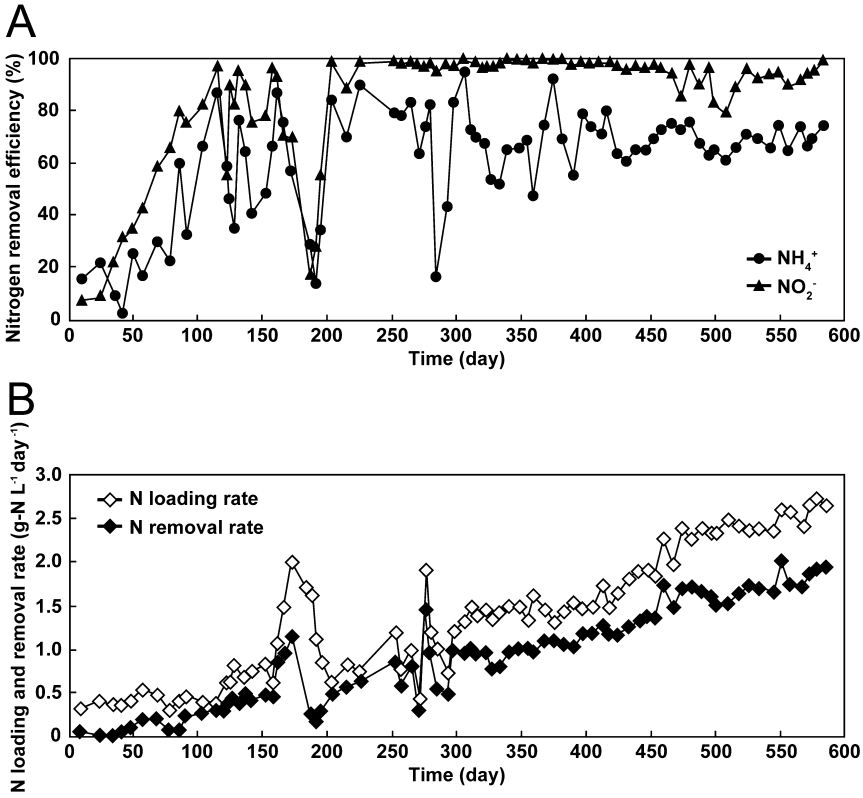

Fig. 1. NH_4^+ and NO_2^- removal efficiency (A) and nitrogen loading rate and removal rate (B) during the experiment.

reported previously, 1:1.32:0.26, for the anammox reaction in a sequencing batch reactor fed synthetic wastewater (30).

The nitrogen removal rate was six to 31 times higher than the values reported in other studies that used a column reactor fed deep sea water containing minerals (20), a column reactor fed a synthetic salt-based medium (12), and sequencing batch reactors fed a sea salt-based medium (35). The high nitrogen removal rate in this study was attributed to the short HRT. Specifically, the 0.3 to 0.9 h HRT employed was much shorter than in other studies (12, 20, 35). We previously reported that a reduction of HRT might prevent nitrite limitation and the accumulation of unknown by-products derived from the anammox reaction (33). Other studies have also found that the performance of anammox reactors was enhanced by stepwise reductions of the HRT (27).

We successfully enriched marine "*Candidatus* Scalindua" species involved in marine anammox using a synthetic marine nutrient medium containing 35 g L^{-1} of artificial sea salt (SEALIFE). Van de Vossenberg *et al.* (35) reported that two species of marine anammox bacteria were successfully enriched when a Red Sea salt-based medium was used. Nakajima *et al.* (20) also established an enrichment culture of marine anammox bacteria using deep sea water. Conversely, the addition of salt (NaCl and KCl) to standard anammox medium (34) did not result in the enrichment of marine anammox bacteria (11). Due to the low growth rate of marine anammox bacteria, we did not attempt to enrich the bacteria using medium that contained individual salts. However, the presence of microelements that are normally contained in sea water such as bromine, strontium, and fluorine may enhance the enrichment. In fact, Kawagoshi *et al.* (12) reported the successful enrichment of "*Candidatus* Scalindua" species using synthetic sea water.

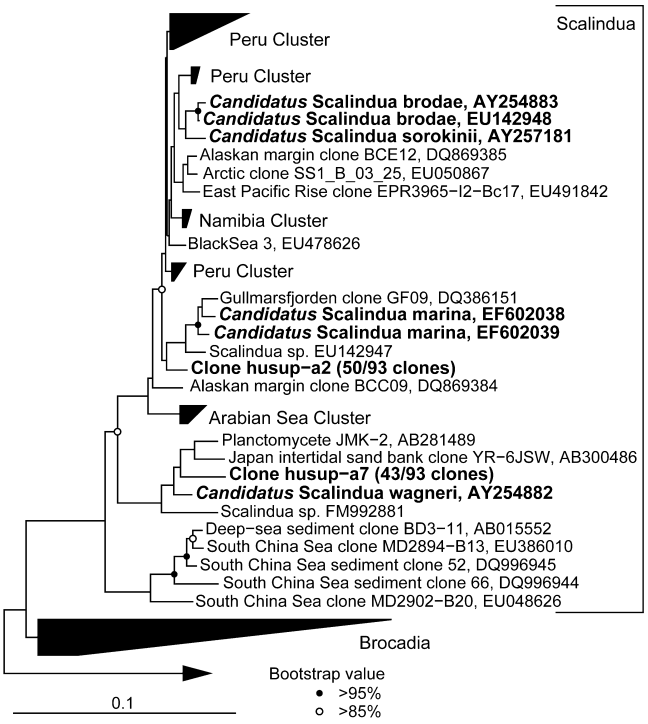

Fig. 2. Phylogenetic tree of clones obtained from the enrichment and related anammox bacteria. The tree was generated by the neighbor-joining method. The scale bar represents the number of nucleotide changes per sequence position. The symbols at each branch point show the bootstrap values obtained with 1,000 resamplings. The GenBank/EMBL/DDBJ accession numbers are also indicated. *Candidatus* genera designations are bracketed on the right. The *Planctomyces maris* (AJ231184) sequence served as the outgroup for rooting the tree. The numbers in parentheses indicate the frequencies of appearance of identical clones among the clones analyzed.

Microbial community structure of the marine anammox bacterial biofilm

To reveal the structure of microbial communities residing in the glass column reactor, three 16S rRNA gene clone libraries, an anammox bacterial clone library, a total bacterial clone library, and an archaeal clone library, were constructed from the biofilm samples taken from the reactor on day 222 at which time a stable anammox reaction after the first pump failure was observed. No chimeric sequences were detected in any of the clone libraries. We obtained 93, 90, and 88 clones from the anammox bacterial clone library, the total bacterial clone library, and archaeal clone library, respectively. The clones were grouped into OTUs based on 97% or higher sequence similarity. We identified at least 12 bacterial phyla and two archaeal phyla including 47 different operational taxonomic units (OTUs) in the marine anammox biofilm. It should be noted that there were no differences in the topology of the two trees generated using the neighbor-joining and the maximum parsimony methods.

In the anammox bacterial phylogenetic tree, 93 clones were classified into two groups (Fig. 2). The sequence of clone husup-a2 was closely related to that of an uncultured *Planctomycetales* bacterium, clone Peru_45, with 98% similarity. This clone was also related to a proposed anammox bacterium, "*Candidatus* Scalindua brodae", with 97% sequence similarity. The sequence similarity of the clone husup-a7 to the closest proposed anammox bacterium, "*Candidatus* Scalindua wagneri", was less than 96%. According to Stackebrandt and Goebel (28), the dominant anammox-like bacterium detected in this study was most likely a novel species of the genus "*Candidatus* Scalindua". The sequence similarity between husup-a2 and husup-a7 was only 93.1%. A distance matrix for the two dominant anammox species indicated that clone husup-a2 belonged to "*Candidatus* Scalindua 1" ("*Candidatus* Scalindua sorokinii/brodae", (37)), whereas clone husup-a7 was closely related to "*Candidatus* Scalindua wagneri". These results clearly indicate that at least two different "*Candidatus* Scalindua" species were present in this reactor.

Ninety clones in the total bacterial phylogenetic tree were classified into 42 OTUs (12 phyla). Figure 3 shows the phylogenetic tree of bacteria without the *Planctomycetes* group. The percentage of bacterial clones other than *Planctomycetes* exceeded 80% of all the clones detected in this study. In contrast to the anammox bacterial clone library, the total bacterial clone library showed a high diversity of clones at the phylum level. These clones were primarily related to uncultured bacterial clones detected in surface sediment from hydrothermal vents, the Arctic Ocean, or hypersaline mats with 87 to 99% sequence similarity; thus, the physiological characteristics of these uncultured bacteria are not clear.

In the archaeal phylogenetic tree, 88 clones were classified into nine groups (Fig. 4). The clones related to

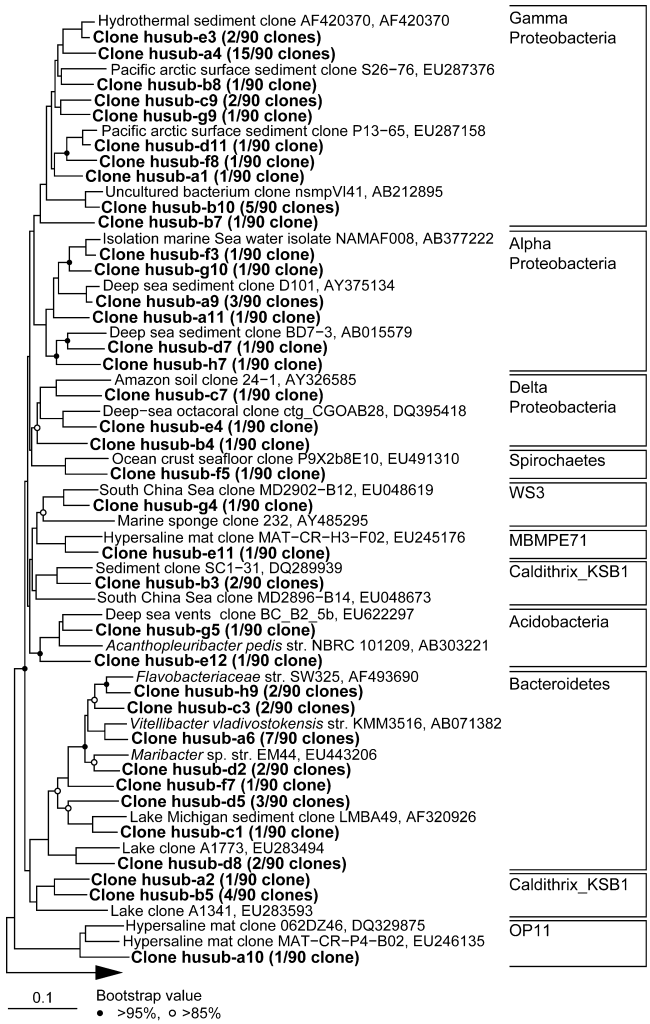

Fig. 3. Phylogenetic tree of clones obtained from the enrichment and related bacteria. The tree was generated by the neighbor-joining method. The scale bar represents the number of nucleotide changes per sequence position. The symbols at each branch point show the bootstrap values obtained with 1,000 resamplings. The GenBank/EMBL/DDBJ accession numbers are also indicated. Division and subdivision designations are bracketed on the right. The *Aquifex aeolicus* (AJ309733) sequence served as the outgroup for rooting the tree. The numbers in parentheses indicate the frequencies of appearance of identical clones among the clones analyzed.

the *Euryarchaeota* and *Crenarchaeota* accounted for 98% and 2% of the total, respectively. All clones except for husua-c3 were affiliated with uncultured archaea with 84 to 94% sequence similarity and belonged to a cluster of clones belonging to deep sea hydrothermal vent euryarchaeotal group 6 (DHVEG-6). This group has primarily been detected in ocean water, coastal water, polar seawater, and deep-sea hydrothermal vents (22, 32). Unfortunately, the ecophysiology and functions of the coexisting *Euryarchaeota* are not yet clear. The sequences of clone husua-c3 and *Nitrosopumilus maritimus* exhibited 99% similarity. *N. maritimus* was recently reported to grow chemolithoautotrophically through the aerobic oxidation of ammonia to nitrite, and is recognized as a member of the ammonia-oxidizing archaea (AOA) (15). AOA have also been found in water and sand samples collected from other bay areas (4, 8).

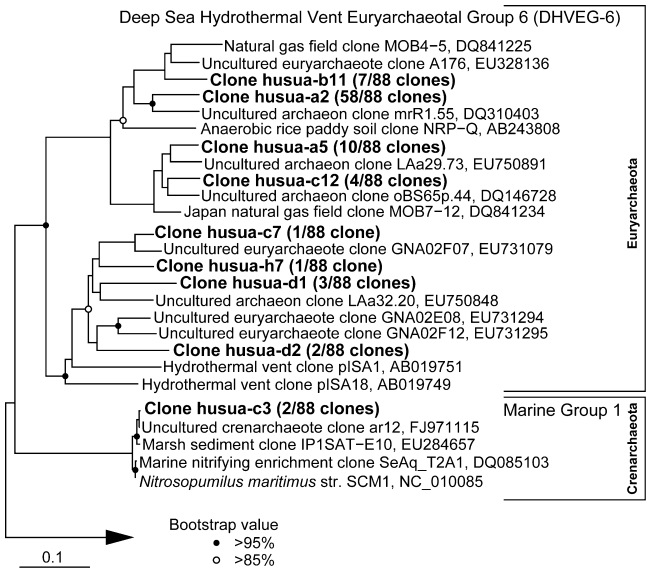

Fig. 4. Phylogenetic tree of clones obtained from the enrichment and related archaea. The tree was generated by the neighbor-joining method. The scale bar represents the number of nucleotide changes per sequence position. The symbols at each branch point show the bootstrap values obtained with 1,000 resamplings. The GenBank/EMBL/DDBJ accession numbers are also indicated. Subdivision designations are bracketed on the right. The *Aquifex aeolicus* (AJ309733) sequence served as the outgroup for rooting the tree. The numbers in parentheses indicate the frequencies of appearance of identical clones among the clones analyzed.

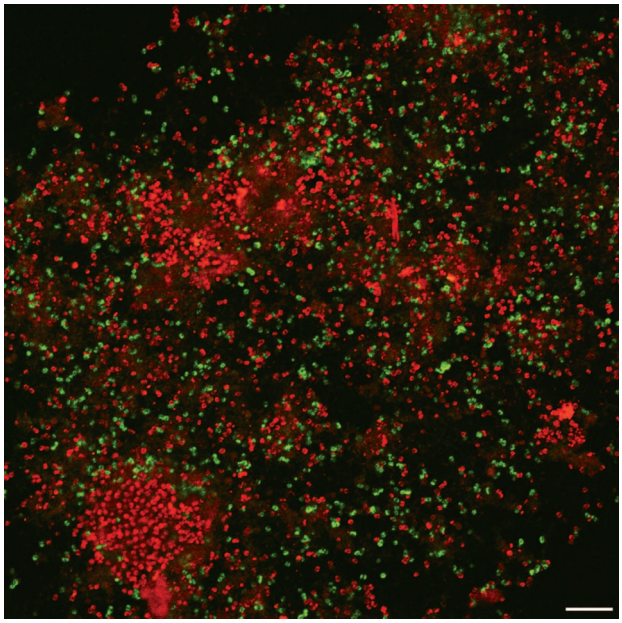

Fig. 5. Confocal laser scanning micrograph of the marine anammox biofilm after homogenization. Green corresponds to the FISH signal of the FITC-labeled probe S-*-Sca-1129-a-A-18. Red corresponds to the FISH signal of the TRITC-labeled probe S-*-Sca-1129-b-A-18. The bar represents 10 μm.

In situ *detection and succession of marine anammox bacteria*

First, we attempted to observe two different "*Candidatus* Scalindua" species by FISH using two newly designed probes, S-*-Sca-1129-a-A-18 and S-*-Sca-1129-b-A-18, which were specific for clones husup-a2 and husup-a7,

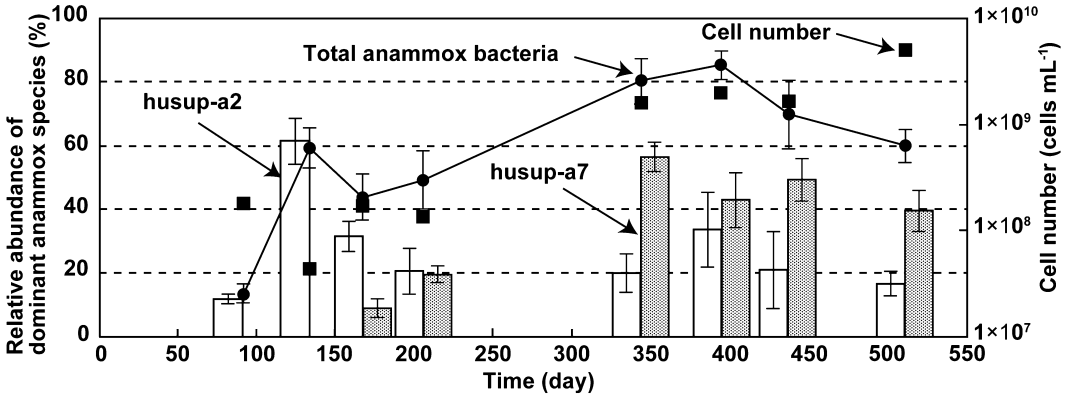

Fig. 6. Relative abundance of the dominant species husup-a2 (white bars) and husup-a7 (gray bars), and all anammox bacteria detected with the probe Amx368 (closed circles). Cell numbers (closed squares) were determined by DAPI counting. Relative abundance is shown as the percentage of each specific probe signal in a microscopic field to the EUB338mix probe signal. The error bars indicate standard deviations.

respectively. Then, changes in the populations of dominant anammox bacteria were determined by FISH. The optimal formamide concentrations were experimentally determined to be 35% for probe S-*-Sca-1129-a-A-18 and 20% for probe S-*-Sca-1129-b-A-18. Figure 5 shows a FISH image of the homogenized biofilm after 433 days of operation. *In situ* hybridization revealed that two different "*Candidatus* Scalindua" species were clearly recognized by the simultaneous use of probes S-*-Sca-1129-a-A-18 and S-*-Sca-1129-b-A-18. The two anammox bacteria enriched in this study showed a typical morphology (approximately 1 μm in diameter and doughnut-shaped) as reported by Kuypers *et al.* (16). No archaea were detected by FISH with the probe ARC915, Cren512, or EURY514, even though many groups of archaea were detected by the phylogenetic analysis. This was likely due to the archaeal population being present at levels below the detection limit of FISH and/or low activity in the biofilm. Another explanation involves the problem with penetration of probes into the cells as FISH was applied without any enzymatic treatments enhancing cell-wall permeability.

Changes in the populations of marine anammox bacteria were determined by FISH using the two newly designed probes (Fig. 6). The population of total anammox bacteria (detected with probe Amx368) as well as total number of cells increased with increasing operation time. Overall, the anammox bacteria accounted for 85.5 ± 4.5% of all the bacteria at day 393. Clone husup-a2 was found to be present throughout the period of operation and to account for 12.7 ± 1.3 to 62.2 ± 7.3% of the total. Clone husup-a7 was not observed until day 166, and comprised 9.8 ± 2.8 to 56.7 ± 4.4% of bacteria. The relative abundance of husup-a2 peaked at day 132, whereas that of husup-a7 peaked at day 342. The composition of total anammox bacteria corresponded with the sum of the compositions of clones husup-a2 and -a7. After day 166, two most abundant species of anammox bacteria coexisted during reactor operation (Fig. 6). One possible explanation for the presence of two species is the operational temperature, which was 20°C, corresponding to the temperature at the location from which the surface sediment sample was collected. Van de Vossenberg *et al.* (35) reported that two "*Candidatus* Scalindua" species were equally abundant in a 15°C culture, whereas one species of "*Candidatus* Scalindua" gradually disappeared in a 23°C culture, while only one species of "*Candidatus* Scalindua" was dominant in a 30°C culture (12). Accordingly, the 20°C reactor temperature employed in this study might not be a selective condition for the two enriched species of "*Candidatus* Scalindua". Thus, the temperature condition is likely to have a strong influence on the appearance of dominant species within "*Candidatus* Scalindua". Accordingly, further study of the population dynamics of the two "*Candidatus* Scalindua" species at different temperatures should be performed.

In conclusion, we successfully enriched marine anammox species using a glass column reactor inoculated with coastal sediment collected from Hiroshima Bay and achieved a total nitrogen removal rate of 2.17 g-N L^{-1} day^{-1}. FISH and phylogenetic analyses revealed that two "*Candidatus* Scalindua" species coexisted in the biofilm and accounted for about 80% of the total bacterial population at days 342 and 393. In addition, a high diversity of uncultured bacteria and archaea coexisted in the marine anammox biofilm. More studies are needed to investigate the characteristics of these marine anammox bacteria, such as the optimal growth temperature, pH, and salinity.

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