

Full Length Research Paper

Enrichment of anammox bacteria from mudflat sediments collected in Tokyo Bay

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We established an enrichment culture of anammox bacteria by seeding a batch reactor with sediments from the Banzu mudflat in Tokyo Bay. The reactor was intermittently fed with an anammox substrate containing NH₄Cl, NaNO₂ and artificial seawater (3.03% salinity) for more than 800 days. An anammox enrichment culture capable of simultaneous reduction of approximately equivalent amounts of ammonium nitrogen and nitrite nitrogen was established. Batch assays of the enrichment culture revealed that anammox activity was similar for all batches at salinities ranging from 1.01 to 3.03%, with lower activity observed at a salinity of 0.02%. We identified three 16S rRNA genes in DNA fragments amplified from the enriched culture. One of these genes, mudflat sediment clone_K01 was highly homologous with *Candidatus* "Kuenenia stuttgartiensis" (identity 97%) being the most abundant (85 out of isolated 94 clones). In most of the studies conducted to date, anammox species belonging to the *Candidatus* "Scalindua" genus have been found to be the dominant species in enrichment cultures obtained from estuarine and marine environments. The results reported here are unusual, in that, a freshwater anammox bacterium, *Candidatus* "K. stuttgartiensis", was the dominant bacterium in a sediment sample from a marine environment.

Key words: Anammox, mudflat sediment, salinity, *Candidatus* "Kuenenia stuttgartiensis", *Candidatus* "Scalindua wagneri".

INTRODUCTION

Tokyo Bay is a typical enclosed coastal bay in Japan. At present, there are seven major mudflats in Tokyo Bay, the largest of which is the Banzu mudflat, with ca. of 1400 ha in size and stretches from the Obitsu River estuary to Tokyo Bay. The term mudflat is used to refer to the zone between the high and low water marks, where mud is

deposited by tides or rivers in sheltered areas or bays. Anaerobic ammonia oxidation (anammox) bacteria are widely distributed in natural ecosystems where they are involved in the removal of nitrogen in estuarine and marine sediments (Amano et al., 2011; Kindaichi et al., 2011; Li et al., 2011). Although, the anammox bacteria in mudflats are considered to contribute to nitrogen removal from enclosed bays; relatively, little information is available on the role of anammox bacteria in mudflat sediments.

We therefore attempted to enrich anammox bacteria

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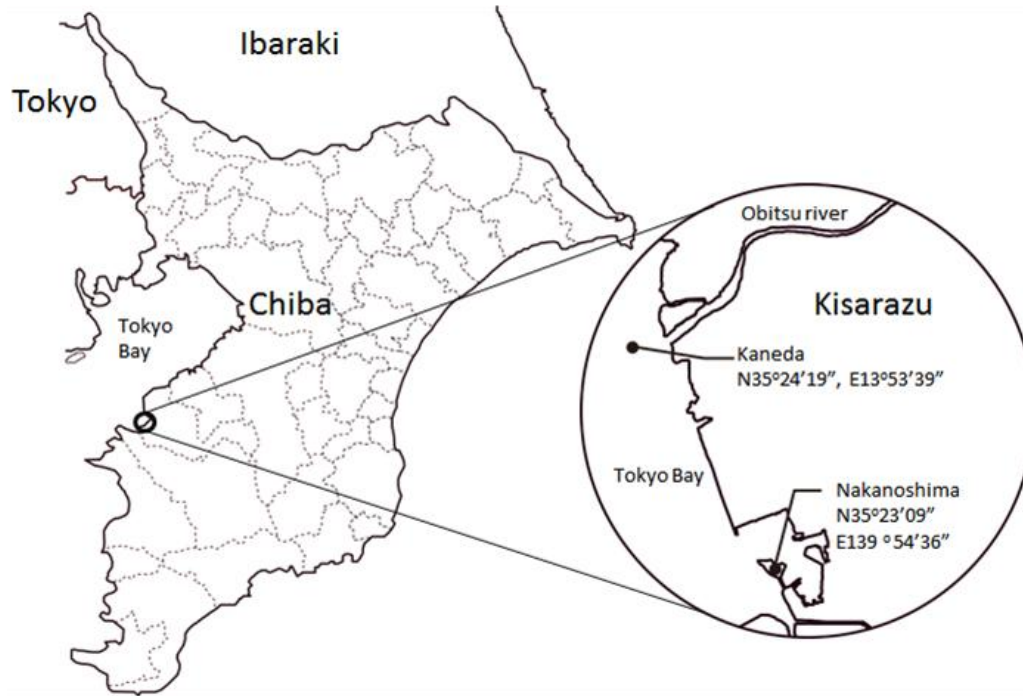


Figure 1. Map of the Banzu mudflat showing the locations of the sample sites.

from mudflat sediments collected in Tokyo Bay. Batch assays were conducted to clarify the relationship between anammox activity and salinity in the enrichment culture and phylogenetic analyses using 16S rRNA gene sequences were performed to clarify the species composition of anammox bacteria in the enrichment.

MATERIALS AND METHODS

Mudflat sediments were collected from sites at Kaneda and Nakanoshima on the Banzu mudflat in Tokyo Bay (Figure 1). Samples were collected from the surface to a depth of 200 mm using a plastic core sampler. The salinities at both sites were 3.4%. To culture the bacteria from the sediments, we previously prepared a mixture of artificial seawater (DAIGO artificial seawater, DAIGO, Japan) and anammox basal medium (Nakajima et al., 2008), which was aerated with nitrogen gas to decrease the dissolved oxygen concentration to <0.1 mg-O₂/L. The salinity of the substrate was 3.03% and the initial concentrations of NH₄⁺-N and NO₂⁻-N were adjusted to 50 mg-N/L. The batch reactor consisted of a 1000 mL flask, gas trap, gas bag and rubber stopper. Then, 100 g of wet sediment was added to the batch reactor containing 400 mL of substrate. After sealing the reactor and flushing with nitrogen gas, the enrichment procedure was initiated in an incubator at 30°C. The reactor was agitated manually once a day to promote contact between the sediment and the substrate. The determination of NH₄⁺-N, NO₂⁻-N and nitrate nitrogen (NO₃⁻-N) was then performed at least once every two weeks. Once almost all of the NH₄⁺-N and NO₂⁻-N had been consumed, the old substrate was replaced with a new substrate.

Once the anammox enrichment culture from the Kaneda site was established and simultaneous reduction of approximately equivalent amounts of NH₄⁺-N and NO₂⁻-N had successfully been achieved, the effect of salinity on the anammox activity of the enriched culture

was investigated in a batch assay (day 721). Using the artificial seawater, the salinity of the substrate was adjusted as follows: 0.02 (typically used for anammox substrate), 1.01, 2.02 and 3.03%. The initial concentrations of NH₄⁺-N and NO₂⁻-N were adjusted to 100 mg-N/L. In a chamber filled with nitrogen gas, 3.5 g (wet weight) of enriched sediment was then transferred from the batch reactor to serum vial bottles (120 mL) containing 25 mL of substrate and the bottles were sealed with butyl rubber stoppers and aluminum caps. To ensure anaerobic conditions, the head space of each bottle was purged with nitrogen gas after transferring the sediments as described above. The bottles were then incubated in a temperature-controlled water bath at 30°C.

The sediment samples used for DNA extraction were collected from the batch reactor on day 742. DNA was extracted from the bacteria in the sediment [3.5 ± 0.01 g (wet weight)] using a FastDNA spin kit for soil (BIO101, Qbiogene Inc., Carlsbad, CA). The 16S rRNA gene fragments were amplified from the extracted total DNA using *Taq* DNA polymerase (Takara Bio Inc., Japan) and the Planctomycetales-specific primer set Pla46f (5'-GGATTAGGCATSCAAGTC-3') (Neef et al., 1998) and Amx820r (5'-AAAACCCCTCTACTTAGTCCC-3') (Schmid et al., 2000). The polymerase chain reaction (PCR) conditions used to target anammox bacteria consisted of initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, with a final extension step of 72°C for 4 min. The PCR products were electrophoresed on a 1% (wt/vol) agarose gel.

PCR products were then ligated into a TOPO vector and transformed into competent cells according to the manufacturer's instructions (TOPO TA PCR cloning; Invitrogen, CA). Nucleotide sequencing was performed using an automatic sequencer (Applied Biosystems 3730xl DNA Analyzer, Applied Biosystems, CA). Sequences from clones with more than 97% sequence similarity were grouped into the same operational taxonomic unit using the similarity matrix program implemented in the Arb software package (Ludwig et al., 2004), and a phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).

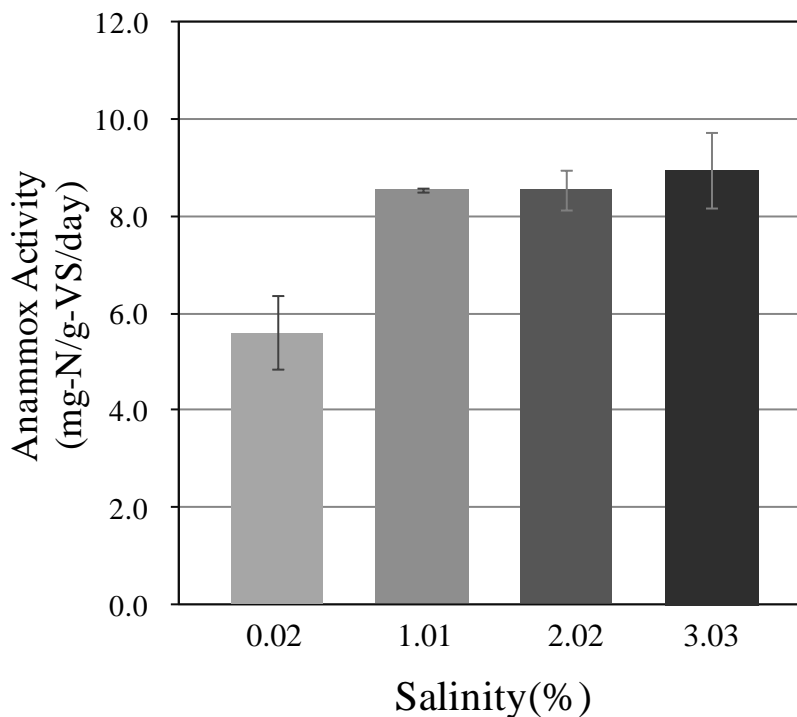


Figure 2. Anammox activity in mudflat sediments as a function of salinity.

RESULTS AND DISCUSSION

The dry solid content of the Kaneda sediments was 78.2% (w/w) and the ash content was 98.4% (w/w). The Kaneda sediments almost completely consumed 50 mg-N/L of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ in the initial 190 days of the experiment. Unfortunately, enrichment of the sediment collected at the Nakanoshima site did not reveal the consumption of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$, even after 250 days of incubation. The reason for the differential distribution of anammox bacteria within the same mudflat has not yet been determined, but a previous study had reported similar disparities in establishing enrichment cultures of anammox bacteria from various kinds of samples (Tsushima et al., 2007). In addition, previous reports have demonstrated almost complete consumption of $\text{NH}_4^+\text{-N}$ and $\text{NO}_2^-\text{-N}$ by enriched cultures of anammox bacteria between 37 to 240 days (Tsushima et al., 2007; Nakajima et al., 2008), which is comparable to our results using sediments from the Kaneda site.

After 721 days of operation, sediment samples were transferred from the reactor and incubated in serum vials to determine maximum anammox activity over salinities ranging from 0.02 to 3.03% (Figure 2). The sediment exhibited a maximum anammox activity of 8.97 mg-N per g of volatile solids (VS) per day (mg-N/g-VS/d) at a salinity of 3.03%. Anammox activity decreased slightly approximately to 95% of the maximum (8.55 to 8.54 mg-N/g-VS/d) at salinities of 1.01 to 2.02% and to 62.5% (5.61 mg-N/g-VS/d) at salinities of 0.02%. The expected

anammox stoichiometry ($\text{NO}_2^-\text{-N} : \text{NH}_4^+\text{-N} = 1.1\text{-}1.3$) was observed in all of the batch assays.

To date, most of the enrichment cultures of anammox bacteria that were collected from estuarine or marine ecosystems consist of anammox bacteria belonging to the "Scalindua" genus (Kartal et al., 2006; Amano et al., 2011; Li et al., 2011). However, after more than 700 days of operation under high salinity conditions, the results of the cloning analysis performed in this study revealed that the freshwater anammox species *Candidatus* "K. stuttgartiensis" was the most dominant species in the enriched sediment. Specifically, 90.4% of the anammox bacterial clones in the sediment belonged to *Ca.* "K. stuttgartiensis" (mudflat sediment clone_K01, 85 of the 94 clones), 8.5% belonged to *Ca.* "Scalindua wagneri" (mudflat sediment clone_K02, 8/94), and 1.1% were related to the trickling filter biofilm clone, KOLL2a (mudflat sediment clone_K03, 1/94) (Figure 3). Interestingly, the "Kuenenia" genus is generally considered to be a freshwater species. To the best of our knowledge, very few studies conducted on anammox bacteria in marine sediments to date have found members of the genus "Kuenenia" to be more prevalent than representatives of the genus "Scalindua" (Kartal et al., 2006; Amano et al., 2011; Li et al., 2011). Thus, the finding that *Ca.* "K. stuttgartiensis" was the dominant bacterium in the enrichment culture produced from the marine sediments appears to be unique.

However, the underlying reasons for why the freshwater anammox bacterium *Ca.* "K. stuttgartiensis"

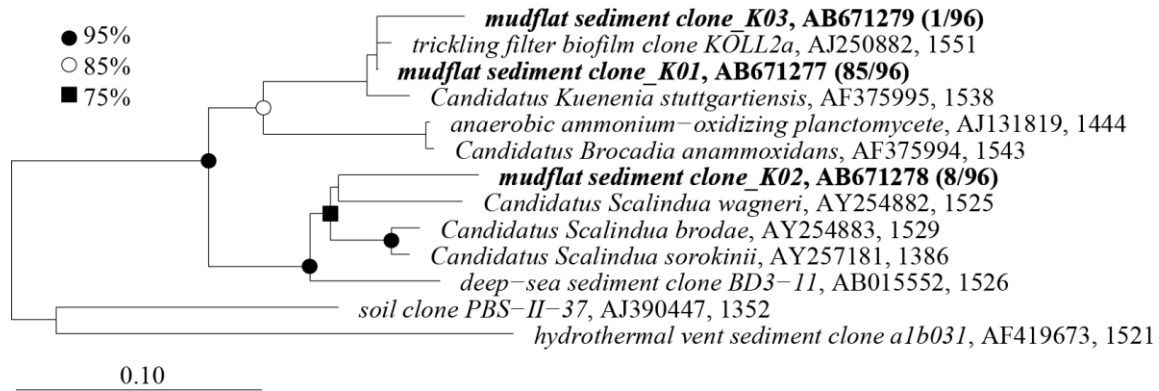


Figure 3. Phylogenetic tree generated based on 16S rRNA sequences of mudflat sediment clones, K01, K02 and K03, and authentic anammox bacteria. Nodes supported by bootstrap values of >75%, >85% and >95% are indicated by solid squares, open circles and solid circles respectively. The bar represents an estimated sequence divergence of 10%.

was dominant in the mudflat sediments is not yet clear. Kartal et al. (2006) inoculated a high salinity anammox reactor with biomass harvested from a freshwater wastewater treatment system. These authors found that freshwater anammox bacteria adapted to salt concentrations as high as 30 g/L. Interestingly, the dominant anammox species isolated in that study was also *Ca. "K. stuttgartiensis"*, which implies that *Ca. "K. stuttgartiensis"* is capable of developing salinity tolerance under long-term enrichment conditions.

The batch assay used in this study to observe maximum anammox activity showed that the freshwater species, *Ca. "K. stuttgartiensis"*, could adapt to salinities as high as 3.03‰ while maintaining anammox activity (Figure 2). Thus, though salinity is an important parameter affecting the growth of bacterial populations in natural ecosystems, sediment salinities as high as 3.03‰ did not confer a competitive advantage upon *Ca. "S. wagneri"* species and allow them to outcompete *Ca. "K. stuttgartiensis"*. This finding implies that salinity tolerance may not be the only factor affecting the niche width of "Scalindua" species. Nonetheless, the observation that 8.5% of the species in the sediment belonged to the genus "Scalindua" indicated that salinity may well be one of the factors contributing to the existence of the members of this genus in the marine environment.

Li et al. (2011) investigated the seasonal dynamics of anammox bacteria in estuary sediments from the Mai Po Nature Reserve in Hong Kong. Their findings showed that while members of "Scalindua" occurred in summer and winter, "Kuenenia" anammox bacteria were only present, sometimes even dominant, at certain sites in the Mai Po Nature Reserve in summer. They concluded that the seasonal dynamics of the anammox bacterial community may have been affected by a combination of anthropogenic, terrestrial and environmental parameters, including NO_x, NH₄⁺, TN, TKN, TP, salinity, and especially temperature. Thus, in order to better understand the

observed variation in the bacterial community structure of marine environments, the relative influence of sampling location and environmental factors should be carefully examined.

Conclusion

We established an enrichment culture of anammox bacteria in a batch reactor that was seeded with sediments from the Banzu mudflat in Tokyo Bay. Batch assays of the enrichment culture revealed that the sediment exhibited a maximum anammox activity at a salinity of 3.03‰, and that anammox activities decreased at salinities of 0.02 to 2.02‰. Cloning analysis revealed that the freshwater anammox species *Ca. "K. stuttgartiensis"* was dominant in the enriched sediments. The results of this study were unusual, in that, *Ca. "K. stuttgartiensis"* was the dominant species in an enrichment culture inoculated with marine sediments.

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