A Search for Anaerobic Ammonia Oxidizing (Anammox) Bacteria in Plum Island Sound Estuarine System

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ABSTRACT

The potential for anaerobic oxidation of ammonia (anammox) in sediments had been explored around the world. In this study, the presence of bacteria responsible for this process was examined in a high salinity and low salinity site in Plum Island Sound Estuarine System, MA. PCR of 16S rRNA gene using Planctomycetale- and anammox-specific primers was employed to detect the presence of this specific group of bacteria. While PCR products of target size (322 bp) were obtained from DNA extracted from all samples at high salinity site and lower section (15-35 cm) of low salinity site, relatively few or no PCR products of target size were obtained from the upper section (0-10 cm) of sediments from low salinity site. Sequence analysis of bacterial clone libraries of low salinity site demonstrated a diverse microbial community, but no sequence related to recognized anammox bacteria was identified.

INTRODUCTION

Classical geochemical model of nitrogen cycle restricts ammonia oxidation to oxic environment or oxic-anoxic interfaces. However, based on the nutrient profiles and thermodynamic calculations, an anaerobic pathway of ammonia oxidation (anammox) had been hypothesized. Such pathway was not discovered until a decade ago in a wastewater treatment system in Delft, the Netherlands (4). Nonetheless, the presence and abundance of this group in the environment was only recently discovered. A recent study indicated that anaerobic ammonia oxidation coupled to nitrate reduction might contribute 67% of the total N₂ production at the continental shelf site at Skagerrak, Denmark (2). Studies of an anoxic natural basin in the Black Sea also demonstrated both anammox activity and the presence of ladderane lipids in the microbial consortia of the particulate organic matter floating in the water column that are unique to anammox at the same range of depths(3).

Molecular analysis of 16S rRNA gene sequences from these sites showed the presence of organisms affiliated with the phylum Planctomycetes. Currently, only three clusters in Planctomycetes have been identified to carry out anammox, including Candidatus “Scalindua”, Candidatus “Brocadia” and Candidatus “Kueneia”. Two clusters of these three were only discovered in the last three years (5). Thus the detection and identification of active anammox organisms in environmental samples along with analysis of environmental profiles will facilitate the discovery of more anammox bacteria.

In this project, PCR specific for detection of anammox bacteria was employed to examine the presence of anammox bacteria in Plum Island Sound Estuarine System. While no direct evidence had been provided for the presence of anammox in this environment, the high concentrations of ammonia and nitrite in deeper sediment of this site indicate the potential presence of anammox bacteria. Moreover, effect of salinity on the presence of anammox was compared.
MATERIALS AND METHODS

Sample location and collection

Two sediment cores of ca. 35 cm were taken from Plum Island Sound Estuarine System (Figure 1) using plastic cylindrical corer with minimal disturbance to the sample. Samples collected from Nelson Island Preserve (High salinity site) were with higher salinity than samples collected from Parker River site (Low salinity site). Cores were sliced at 5-cm intervals and stored in the dark at -4 °C until further analysis. Analysis was carried out on samples from the undisturbed middle section of the core slices.

DNA extraction

Nucleic acids were extracted from 100 -200 mg of the undisturbed sediment core with Epicenter SoilMaster™ DNA Extraction Kit (Epicenter Biotechnologies, Madison, WI). 200 µL of extracted nucleic acids were first concentrated to 100 µL using Microcon® Centrifugal Filter Units (Millipore, Billerica, MA) and then further purified using Wizard® DNA Clean-Up System (Promega, Madison, WI). Gel electrophoresis had been carried out to assess the presence of genomic nucleic acids in the samples. The purified DNA templates were resuspended in 50 µL of RNase-free sterile water and stored in -20 °C.

PCR amplification of environmental 16S rRNA genes

Purified extracted DNA were amplified using 16S rRNA primer sets specific for bacteria, planctomycetes and anammox strains (Table1).While direct PCR was employed for all three sets of primers, PCR products from Planctomycetale-specific reactions was also reamplified for targeting anammox strains. The 25- µL PCR reaction mixtures contain 2.5 µL of 10X MgCl2 free buffer, 2 mM of MgCl2, 0.75 U of Taq Polymerase, 2 mM of deoxynucleoside triphosphate (dNTPs) (Promega, Madison, WI), 0.25 µM of all primers and 0.5 µL of BSA . 1 µL of DNA templates or PCR products were used in each reaction. DNA of Planctomyce maris was contributed by the laboratory of Dr. Katrina Edwards (WHOI) to be used as the positive control.
of PCR targeting *Planctomycetes*. This strain, however, is not known to carry out anammox reaction. The thermal cycle conditions for each set of primers are described in Table 1.

### Table 1. PCR primers and thermal profiles

<table>
<thead>
<tr>
<th>Primer Set (fragment length)</th>
<th>Target Group</th>
<th>PCR approach</th>
<th>Initial Denaturation temp (°C) [sec]</th>
<th>Annealing temp (°C) [sec]</th>
<th>Extension temp (°C) [time]</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>8f; 1492r (1.4 kb)</td>
<td>Eubacteria</td>
<td>Direct</td>
<td>95 [30]</td>
<td>50 [30]</td>
<td>72 [90]</td>
<td>-</td>
</tr>
<tr>
<td>Pla46; 1392r (1.3 kb)</td>
<td>Planctomycetes</td>
<td>Direct</td>
<td>94 [50]</td>
<td>56 [60]</td>
<td>72 [110]</td>
<td>(6)</td>
</tr>
<tr>
<td>Pla46; Amx368 (323 bp)</td>
<td>Anammox</td>
<td>Direct, Nested</td>
<td>94 [50]</td>
<td>56 [60]</td>
<td>72 [110]</td>
<td>(5)</td>
</tr>
</tbody>
</table>

Note: All PCR started with an initial denaturation at 95°C for 5 minutes and 35 cycles of the thermal profile listed above, followed by a 7-minute final extension at 72°C.

**Cloning, sequencing and database analysis of environmental 16S rDNA**

PCR-amplified DNA fragments using bacterial primers (8F and 1492R) were introduced into a pCR2A vector and transformed into *Escherichia coli* using a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). Sequencing was carried out by using the ABI 3730XL capillary sequencer at the Keck facility of the Josephine Bay Paul Center (Marine Biological Laboratory, Woods Hole, MA). Partial DNA sequences were aligned and analyzed by using ARB software. Phylogenetic relationships were calculated by using maximum-parsimony analysis with 1,000 times bootstrap resampling.

**Activity Assays**

Activity assays were prepared in 175-mL serum bottles containing approximately 80-mL of sea water medium (Table 2), with ammonium as electron donor and nitrite as electron acceptor. Sediments from 20, 30 and 35 cm depths of both sites were used to inoculate these media. Assays inoculated with autoclaved sediments or without any sediment were used as controls. Headspace of each bottle was flushed with N₂ (80%)/CO₂ (20%) and sealed with gastight butyl rubber stoppers. All assays were prepared in triplicates and shaken (150 rpm) in the dark at 37 °C. Samples were collected from the liquid phase at the beginning, day 3 and day 9 and stored at -20 °C prior to analysis.

### Table 2. Media used for activity assay (Please refer to the handout “Enrichment, Modular Media Designs, and Isolation” for detailed recipe of each component). This recipe was modified after discussion with Jerad Leadbetter and (1)

<table>
<thead>
<tr>
<th>Components</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seawater</td>
<td>1 X</td>
</tr>
<tr>
<td>EDTA-Chelated Trace Elements</td>
<td>1 X</td>
</tr>
<tr>
<td>Phosphate</td>
<td>1 mM</td>
</tr>
<tr>
<td>Sodium sulfate</td>
<td>0.25 mM</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>3 mM</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>6 mM</td>
</tr>
<tr>
<td>12-vitamin</td>
<td>1 X</td>
</tr>
<tr>
<td>Vitamin B₁₂</td>
<td>1 X</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>5 mM</td>
</tr>
</tbody>
</table>

**Analysis of ammonia, nitrite and nitrate**

The concentrations of total ammonia (NH₃ and NH₄⁺), nitrite and nitrate in the liquid phase of the assays were analyzed spectrophotometrically (Cary model 50 UV/Visible) by using the salicylate-nitroprusside and sulfanilamide-naphthylenediamine methods, respectively.
RESULTS

PCR amplification.

Eight DNA extractions were set up for each site. Gel electrophoresis of crude nucleic acid extracts demonstrated successful DNA extraction. Since anammox bacteria was expected to be present only in the anoxic zone, only DNAs from the lower section (20, 25, 30 and 35 cm) of the sediment cores were first examined in PCR. PCR amplification of the crude extracts was not successful using the bacterial- and Planctomycetales-specific primers. After the concentration and purification step, bacterial PCR amplifications were only successful for two samples from the low salinity sites (LS30, LS35). Second purification step was carried out for all samples. PCR products could be obtained using Planctomycetale-specific, but not bacterial-specific primers for these samples. While nested PCR amplifications with anammox-specific primers yielded stronger bands between 300-400 bp in gel electrophoresis (Figure 2a), direct PCR amplification of DNAs from 20 cm and 30 cm depth of high salinity site and 25, 30 and 35 cm depth of low salinity site also generated target bands (Figure 2b).

Figure 2. (a) Gel electrophoresis of nested PCR with Planctomycetale-specific primers for the first stage of PCR and anammox-specific primers for the secondary PCR; (b) Gel electrophoresis of direct PCR products with anammox-specific 16S rRNA primers. 100-bp ladder was used. -: negative control; Lanes 1-3: PCR products from high salinity site (HS) at different depths. 1: 20 cm; 2: 30 cm; 3: 35 cm; Lanes 4-7: PCR products from low salinity site (LS) at different depths. 4: 20 cm; 5: 25 cm; 6: 30 cm; 7: 35 cm. Lane 8: Planctomyce maris

Due to the strong signals of PCR products between 300-400 bp obtained from samples of the lower section, PCR amplifications of the DNAs extracted from the upper part of the cores were also carried out. Moreover, due to the high turbidity of the activity assays, nucleic acids were also extracted from them and analyzed in PCR. For the PCR using Planctomycetale-specific primers, products could be obtained from all samples. However, relatively weak bands were obtained from samples of the upper sections (0, 5, 10 cm) of low salinity sediment cores (Figure 3). Sizes of products from nested (Figure 4) and direct PCR (data not shown) with anammox-specific primers were within the target range (300-400 bp). A strong signal was obtained from nested PCR of DNA from activity assay inoculated with sediments from 30 cm deep of high salinity site. Again, relatively weak signals were obtained from samples of the upper core of low salinity sediment cores.
In all PCR amplifications, no product was obtained from negative control tubes. Also, no products (322 bp) were obtained from *Planctomyce maris* in the nested and direct PCR using anammox-specific primers.

Figure 3. Gel electrophoresis of PCR products with *Planctomycetales*-specific 16S rRNA primers. 100-bp ladder was used. :: negative control; Lanes 1-6 PCR products from activity assays. Labels: HS: High Salinity; LS: Low Salinity; e.g. HS20-2 refers to 2nd replicate of assays inoculated from 20 cm depth at high salinity site. 1: HS20-2; 2: HS30-1; 3: HS35-3; 4: LS20-1; 5: LS30-2; 6: LS35-1; Lanes 7-10: PCR products from high salinity site at different depths. 7: 0 cm; 8: 5 cm; 9: 10 cm; 10: 15 cm. Lanes 11-14: PCR products from low salinity site at different depths. 11: 0 cm; 12: 5 cm; 13: 10 cm; 14: 15 cm. Lane 15: *Planctomyce maris*;

Figure 4. Gel electrophoresis of nested PCR products with anammox-specific 16S rRNA primers. 100-bp ladder was used. :: negative control; Lanes 1-6 PCR products from enrichments. Labels: HS: High Salinity; LS: Low Salinity; e.g. HS20-2 refers to 2nd replicate of assays inoculated from 20 cm depth at high salinity site. 1: HS20-2; 2: HS30-1; 3: HS35-3; 4: LS20-1; 5: LS30-2; 6: LS35-1; Lane 7: *Planctomyce maris*; Lanes 8-11:
Clone library analysis

PCR products from samples LS30 and LS35 using bacterial primers were sent for sequencing. Among 70 clones from both samples, none of them were associated with anammox organism and only 1 clone is associated with Planctomycetale based on the current database. The phylogenetic tree also demonstrated that there was no distinctive difference between the community at 30 cm and 35 cm of low salinity sediment (Figure 5). As demonstrated in other sediment and soil community study, the microbial community at both depths is very diverse. 26 clones are associated with Delta-Proteobacteria, 8 clones are affiliated with Nitrospirae, 7 clones associated with Bacteroidetes, and 7 clones are affiliated with Acidobacteria. Other clones are distributed over the phylogenetic tree. No specific characteristics of microbial community could be observed.

Activity Assays

Although calibration curves could be obtained for both methods, these colorimetric methods could not be applied to analyze ammonia, nitrite and nitrate of the samples. The measured values for ammonia was below detection limit for all samples, including non-inoculation and autoclaved controls, while the measured nitrite concentrations of the media represented only ~20% of the actual added concentration. Only 30-40% recovery of nitrite could be detected in these samples after spikes of nitrite standards (based on Laurie’s analysis). Therefore, results from these assays were not valid for further analysis.

DISCUSSION

Ammonia oxidation is traditionally associated with oxic zone of the sediment. Even though anaerobic ammonia oxidation had been discovered in some wastewater treatment systems, their significance in nitrogen cycle in the nature is often ignored. Therefore, the aim of this study was to determine if anammox bacteria was present in different depths of sediment cores and effect of salinity on their presence.

Anaerobic ammonia oxidation had been anticipated many years ago based on chemical analysis data, anammox bacteria were only recently discovered. Due to the limited information of this group of organisms, PCR amplification with 16S rRNA gene-targeted primers and subsequent phylogenetic analysis of the products would be essential for detecting anammox in the environment. Moreover, their slow growth rate imply the relatively long period for enrichment, therefore slows down the acquisition of pure cultures and the study of their physiology. In this study, both nested and direct PCR amplifications using anammox-specific primer set Pla46 and Amx368 were applied to detect the presence of anammox bacteria in the sediments with high and low salinity. While anammox was only expected to be present in the anoxic zone, the results from this study demonstrated that they might be present in the aerobic zone as well.
Figure 5. Maximum parsimony phylogenetic tree of 16S rRNA gene sequences from sediments at 30 cm (red) and 35 cm (blue) of Parker River site in Plum Island Sound Estuarine System together with sequences from closely related environmental clones (black).
Also, while previous studies often required nested approach for amplifying anammox-specific 16S rRNA gene in wastewater treatment systems or the environment, PCR products could be obtained from direct PCR approach in this study, indicating their abundance might be higher than previously anticipated. In spite of the possibility of the ubiquity of anammox in the environment, special caution should be taken to make such conclusion from this study. First, although the pair of primers used in this study had been claimed to be very specific to anammox (5), direct sequencing of the PCR products had not been carried out to identify the bacteria responsible for the products. Since anammox is still underrepresented in the general 16S rRNA sequence databases, clone library using the anammox specific primers maybe required to understand the diversity of this group of microbes. In addition, the specificity of the primers should be further evaluated after the expansion of the sequence database such that more sequences from anammox-associated clusters would be included. Moreover, different annealing temperatures should be tested to determine the stringency of the thermal profile used in this study.

On the other hand, the results of the activity assays confirmed the necessity of applying stable isotopic techniques for detecting anammox activity. As demonstrated in this study, inhibitory substances, such as polyvalent ions, in the system could have affected the colorimetric method to a significant degree. Moreover, the presence of the organic matter in the initial inoculation might promote growth of other anaerobic heterotrophs, which would use ammonia as nitrogen in the system and therefore affect the interpretation of results from this study.

While being cautious in interpreting results from this study, the significance of anammox in geochemical cycling in the environment should not be underestimated. Design of a better media that could enhance the growth of anammox would also be important for isolation of more anammox. Be skeptical but open-minded will be essential to better understand the metabolic pathways and niches of anammox, as well as discovery of new microbe, in the environment.

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REFERENCES


