Successful Enrichment of Rarely Found Candidatus Anammoxoglobus propionicus from Leachate Sludge

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Bacteria that mediate the anaerobic oxidation of ammonium (anammox) have been detected in natural ecosystems, as well as various wastewater treatment systems. In this study, sludge from a particular landfill leachate anaerobic treatment system was selected as the incubation seed for anammox microorganism enrichment owing to its possible anammox activity. Transmission electron microscopy observation, denaturing gradient gel electrophoresis analysis, and cloning/sequencing techniques were applied to identify the diversity of anammox microorganisms throughout the incubation. During the early stage of operation, the diversity of anammox microorganisms was similar to the original complex microbes in the seed sludge. However, as incubation time increased, the anammox microorganism diversity within the system that was originally dominated by Candidatus (Ca.) Brocadia sp. was replaced by Ca. Anammoxoglobus propionicus. The domination of Ca. Anammoxoglobus propionicus produced a stable removal of ammonia (70 mg-N/l) and nitrite (90 mg-N/l), and the total nitrogen removal efficiency was maintained at nearly 95%. The fluorescence in situ hybridization results showed that Ca. Anammoxoglobus propionicus was successfully enriched from 1.8 ± 0.6% initially to 65 ± 5% after 481 days of operation. Therefore, the present results demonstrated the feasibility of enriching Ca. Anammoxoglobus propionicus from leachate sludge, even though the original cell count was extremely low. Application of this seldom found anammox organism could offer an alternative to current ammonia-nitrogen treatment.

**Keywords:** Anammox, Ca. Anammoxoglobus propionicus, PCR-DGGE, primer, TEM

**Introduction**

Anaerobic ammonium oxidation (anammox) was recently developed as a biological wastewater treatment technology and has also become a potential solution for the removal of nitrogenous contaminants from wastewater [8, 15]. Microorganisms that catalyze this reaction have been identified as anammox bacteria, which are chemolithoautotrophic microorganisms that conserve energy by oxidizing ammonium with nitrite as an electron acceptor under anaerobic conditions. Anammox bacteria compose a distinct, deep branching phylogenetic group in the order Planctomycetales; five genera of anammox bacteria have already been described and provisionally named Candidatus (Ca.) Anammoxoglobus propionicus, Ca. Brocadia anammoxidans, Ca. Brocadia fulgisa, Ca. Jettenia asiatica, Ca. Kuenenia stuttgartiensis, Ca. Scalindua wagleri, Ca. Scalindua sorokinii, and Ca. Scalindua brodae [2, 11, 20]. Anammox bacteria are characterized by their extremely slow growth rate; the doubling time of anammox bacteria ranges from 5 to 30 days [17, 25, 28]. Recent studies have also revealed that some of these microorganisms can use organic acids as electron donor and undergo anammox [6, 11, 26]. Anammox bacteria can be found in many ecosystems, including agricultural soils, contaminated porous aquifers, freshwater and marine sediments, hot springs, lakes, lakeshores, marshes, oxygen minimum zones, and wastewater treatment plants [7, 9, 13, 14, 18, 23]. Yet, different anammox species are rarely found in the same ecosystem, and there are large phylogenetic distances between different
species as members of *Planctomycetales* [11]. For example, *Ca. Brocadia anammoxidans* and *Ca. Kuenenia stuttgartiensis* were found to coexist in a leachate-treating rotating disk contactor, and approximately 15% of the nitrite utilized during autotrophic growth was converted to nitrate [5]. Xiao *et al.* [34] also confirmed that *Ca. Kuenenia stuttgartiensis* was an anammox microorganism thriving in a landfill leachate treated in a sequencing batch biofilm reactor. Yapsakli *et al.* [35] subsequently established that *Ca. Kuenenia stuttgartiensis* is the major nitrogen converter in leachate treatment plants. Daverey *et al.* [4] also demonstrated the simultaneous occurrence of partial nitrification, anaerobic ammonium oxidation, and denitrification in a landfill leachate treated under a single partially aerated bioreactor, and indicated that *Ca. Kuenenia stuttgartiensis* was one of the dominant species in the reactor.

In our previous study, we monitored the composition of biogas from a leachate treatment anaerobic tank located in central Taiwan and found high concentrations of N\(_2\) in its off-gas aside from methane. This rare phenomenon prompted our interest to examine the possible existence of anammox bacteria in that particular system. Initial results confirmed the existence of anammox microorganisms, but with low cell counts (data not shown). Accordingly, in the present study, a laboratory-scale batch reactor designed to operate in an autotrophic anammox mode was operated to enrich the anammox microorganisms using this leachate sludge as the initial seed. The anammox bacteria in this enrichment were characterized, and their physiological characteristics compared with those of previously reported anammox bacteria. A regular water quality analysis, denaturing gradient gel electrophoresis (DGGE), cloning, and transmission electron microscopy (TEM) were also applied to explore the microbial community composition.

**Materials and Methods**

**Enrichment and Cultivation of Anammox Bacteria**

A laboratory-scale batch-mode reactor (blood vase, 900 ml culture volume, 100 ml headspace) was used to enrich and culture the anammox bacteria. Sludge collected from a local landfill leachate anaerobic treatment system (treating 500 mg-N/l NH\(_4\)\(^+-N\)) was used as the seeding. This landfill is used for the disposal of domestic wastes, yet unusually high concentrations of nitrogen gas have been measured in its anaerobic tank off-gas, indicating potential anammox activity. For each batch, the reactor was filled with 900 ml of a mineral medium consisting of (NH\(_4\))\(_2\)SO\(_4\) (0.778 g), NaNO\(_3\) (0.739 g), KHCO\(_3\) (1.25 g), NaH\(_2\)PO\(_4\) (0.06 g), MgSO\(_4\)\(\cdot\)7H\(_2\)O (0.2 g), CaCl\(_2\)\(\cdot\)2H\(_2\)O (0.3 g), FeSO\(_4\) (0.00625 g), EDTA (0.00625 g), HCl (1 M, 1 ml), and 1 ml of a trace element solution II [28] per liter of demineralized water. Once the gas production for each batch operation stopped completely, a fresh medium was added to replace the used medium without wasting any biomass. Overall, each batch lasted for an average of 20 days. The headspace was backfilled at 25 ml/min with 95% Ar-5% CO\(_2\) for 10 min to maintain anoxic conditions. The pressure of the headspace was maintained slightly higher than atmospheric pressure. The reactor was then sealed with silicone stoppers. The pH was set at 7 to 8 and then measured and adjusted with hydrochloric acid. The temperature was not controlled and set at room temperature (27°C to 30°C). The concentrations of NH\(_4\)\(^+-N\), NO\(_2\)\(^--N\), and NO\(_3\)\(^--N\) were measured using colorimetric methods according to standard procedures [3].

**Fluorescence In Situ Hybridization (FISH)**

For the FISH analysis, this study used a Cy3-labeled oligonucleotide probe targeting *Ca. Anammoxoglobus propionicus* to investigate the anammox bacteria. The hybridizations with fluorescent probes were performed as described by Zilles *et al.* [36]. The total cell counts were determined by DAPI staining, plus a probe S*-Apr-0820-a-A-21 targeting *Ca. Anammoxoglobus propionicus* and 40% formamide concentration were used for the hybridization experiment [11]. A total of ten FISH images were taken of the same hybridization sample to determine the average ratio of anammox bacteria to total bacteria.

**DNA Extraction, Polymerase Chain Reaction (PCR), and DGGE**

The total DNA was extracted from each enrichment culture sample (approximately 1 ml) using an UltraClean Soil DNA Isolation Kit (MOBIO, Solana Beach, USA), following the manufacturer’s instructions. The 16S rRNA gene fragments from the extracted total DNA were then amplified with *Tag* DNA polymerase (GoTaq Green Master Mix; Promega, Madison, USA) using the

<table>
<thead>
<tr>
<th>Primer</th>
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<th>Sequence (5’-3’)</th>
<th>Target site*</th>
<th>References</th>
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<td>All anammox bacteria</td>
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<td>368-385</td>
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<tr>
<td>Amx368F</td>
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<td>AAAACCCCTCTACTTAGTGCCC</td>
<td>799-820</td>
<td>[20]</td>
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*16S rRNA position according to *Escherichia coli* numbering.