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Anaerobic Ammonia-Oxidizing Bacteria and Related Activity in Baltimore Inner Harbor Sediment†

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The discovery of bacteria capable of anaerobic ammonia oxidation (anammox) has generated interest in understanding the activity, diversity, and distribution of these bacteria in the environment. In this study anammox activity in sediment samples obtained from the Inner Harbor of Baltimore, Md., was detected by ^{15}N tracer assays. Anammox-specific oligonucleotide primer sets were used to screen a *Planctomycetales*-specific 16S rRNA gene library generated from sediment DNA preparations, and four new anammox bacterial sequences were identified. Three of these sequences form a cohesive new branch of the anammox group, and the fourth sequence branches separately from this group. Denaturing gradient gel electrophoresis analysis of sediment incubated with anammox-specific media confirmed the presence of the four anammox-related 16S rRNA gene sequences. Evidence for the presence of anammox bacteria in Inner Harbor sediment was also obtained by using an anammox-specific probe in fluorescence in situ hybridization studies. To our knowledge, this is the first report of anammox activity and related bacterial 16S rRNA gene sequences from the Chesapeake Bay basin area, and the results suggest that this pathway plays an important role in the nitrogen cycle of this estuarine environment. Furthermore, the presence of these bacteria and their activity in sediment strengthen the contention that anammox-related *Plactomycetales* are globally distributed.

Anaerobic ammonia oxidation (anammox) is a newly discovered pathway of the microbial nitrogen cycle that allows ammonia to be oxidized by nitrite or nitrate under anoxic conditions (30). Carried out by autotrophic bacteria, anammox allows the use of ammonia as an electron donor in anoxic environments. The anammox reaction occurs in an organelle-like compartment called the anammoxosome, in which ammonia is oxidized via hydrazine (N_2H_4) and hydroxylamine (NH_2OH) intermediates (21). These unique bacteria have been found to have a cell wall that lacks peptidoglycan, and they contain intracytoplasmic compartments that are characteristic of the order *Planctomycetales* (16, 30).

The geographical distribution and importance of the anammox process to the global nitrogen cycle are of great interest and are currently being evaluated by several research groups (6, 15, 34). Studies with marine sediment samples have demonstrated the presence of significant anammox activity that may be responsible for as much as 67% of the total nitrogen gas formed in locations such as the continental shelf site at Skagerrak, Denmark (33). The anammox communities of these samples were found to include *Planctomycetales*, but no phylogenetic similarity was found to 16S rRNA gene sequences of known anammox bacteria. Recent studies of an anoxic natural basin in the Black Sea and of the Costa Rica shoreline (6, 15) have 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-capable *Planctomycetales* (26). Phylogenetic analyses of 16S rRNA gene sequences of anammox microorganisms from the Black Sea revealed 87.9% similarity to *Kuenenia stuttgartiensis* and 87.6% similarity to *Brocadia anammoxidans*, two anammox bacteria from wastewater treatment plants (29). Specific 16S rRNA gene probes designed from these anammox bacteria, Amx820 and Amx1240 (29), however, have failed to detect anammox bacteria in environmental samples (33).

The abundance of anammox bacteria in wastewater treatment plants has been demonstrated (8, 9), and most of the known anammox isolates have been found to be associated with wastewater treatment environments. Two new anammox-related *Planctomycetales*, *Scalindua brodae* and *Scalindua wagneri*, were recently discovered in association with the bacterial consortia of a treatment plant located in Pitsea (United Kingdom) (24). The presence of these anammox-related bacteria was established by electron microscopy and lipid analysis, although their 16S rRNA gene sequences could not be amplified by using the anammox-specific Amx820 primer. The failure of the Amx820 primer to detect putative anammox bacteria when functional activity was found indicates that this group may be quite diverse at the 16S rRNA gene level.

In the present study we focused on examining sediment from the Inner Harbor of Baltimore, Md., for the presence and activity of anammox bacteria. The Inner Harbor is a polluted site that serves as a conduit to the Chesapeake Bay, an estuary in which the nitrogen concentration has increased dramatically over the last few decades (3). This increase in the nitrogen concentration is primarily due to urban development and overuse of nitrogen-based fertilizers. Years of urban and industrial pollution have contaminated the Inner Harbor floor with high concentrations of organic and inorganic compounds that are

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slowly degraded, releasing nitrogen and other compounds into the water column (2).

In this study we report evidence for anammox activity in Baltimore Inner Harbor sediment. Furthermore, amplification of 16S rRNA gene sequences of anammox-related *Planctomycetales* from sediment DNA preparations revealed the presence of novel anammox bacteria, increasing the diversity of this important group. Understanding the activities and roles played by the pathways of the microbial nitrogen cycle in the Chesapeake Bay water column and sediments, including the Inner Harbor, is essential for obtaining valid nitrogen budgets, predicting future crises, and ultimately using these processes for bioremediation purposes.

MATERIALS AND METHODS

Sediment collection and anammox bacterium enrichment. Sediment was collected with a petite Ponar grab sampler from a subsurface depth of 9.1 m in the Inner Harbor of Baltimore, Md. (39°16.8′N, 76°36.1′W) (5). The salinity of the water column immediately above the sediments was 10 ppt at the time of sampling. Samples were stored at 4°C until they were examined. Five grams of sediment was incubated in a 160-ml glass bottle filled with 150 ml of anammox-specific medium (9). The bottles were spiked with 0.7 mmol of ammonia and nitrite, flushed with 99.99% nitrogen gas, sealed with gas-tight butyl rubber stoppers, and placed in an incubator at 37°C for 3 months with shaking; once the ammonia and nitrite were consumed, periodic additions were necessary to replenish these substrates.

Anammox activity. Incubations of 1 g (fresh weight) of sediment with ^{15}N-labeled and unlabeled ammonia and nitrite (0.7 mmol) were carried out in 160-ml glass bottles containing 150 ml of fresh medium. The bottles were flushed with 99.99% nitrogen gas, sealed with gas-tight butyl rubber stoppers, and placed in the dark in a shaking incubator at 37°C. Samples of the liquid phase and headspace gas were periodically collected and analyzed for ammonia, nitrite, $^{29}N_2$, and $^{30}N_2$.

Analysis of ammonia, nitrite, $^{29}N_2$, and $^{30}N_2$. The total ammonia (NH_3 and NH_4^+) was determined by the hypochlorite oxidation reaction as described by Scheiner (22). Nitrite was measured by the sulfanilamide reaction as described by Strickland and Parsons (28). Bottle headspace gas was sampled for the isotopic N_2 combination ($^{29}N_2$ and $^{30}N_2$) by first shaking the bottle to establish equilibrium between the water and headspace N_2 and then injecting 10 μl into a Hewlett-Packard gas chromatograph-mass spectrometer column (HP-5MS) by using a gas-tight syringe. $^{29}N_2$ and $^{30}N_2$ concentrations were determined by calculating their isotopic ratio masses as excess above the natural values in the bottle headspace as described by Thamdrup and Dalsgaard (32).

Extraction of DNA and PCR amplification. DNA from fresh sediment or enriched cultures was extracted as described previously (12). Briefly, 1-ml aliquots of samples were subjected to bead beating and phenol-chloroform extractions, followed by purification by electrophoresis through a 1.3% (wt/vol) low-melting-point agarose gel containing 2% (wt/vol) soluble polyvinylpyrrolidone. Chromosomal DNA was excised from the gel and was recovered by using a Promega Wizard PCR Prep kit (Promega, Madison, Wis.) according to the manufacturer's instructions.

To detect anammox bacteria in sediments, a sequential PCR approach was employed. First, amplification of *Planctomycetales*-specific 16S rRNA genes was done by using the Pla46 oligonucleotide primer (5′-GAC TTG CAT GCC TAA TCC-3′) (17) with the universal bacterial primer 1392r (5′-ACG GGC GGT GTG TAC-3′) (11). Following this an anammox-specific PCR was performed by using Pla46 and either Amx820 (5′-AAA ACC CCT CTA CTT AGT GCCC-3′) or Amx1240 (5′-TTT AGC ATC CCT TTG TAC CAA CC-3′) (29). Amplification of 16S rRNA gene sequences for denaturing gradient gel electrophoresis (DGGE) was performed by using universal bacterium-specific 16S rRNA gene primer 1055f (5′-TGG CTG TCG TCA GCT-3′) and universal bacterial GC clamp primer 1392rGC (5′-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC ACG GGC GGT GTG TAC-3′) (11) with either sediment DNA or the amplified anammox-related *Planctomycetales*-specific PCR product as the template. The PCR mixtures (50 μl) contained 5 μl of 10× PCR buffer, 4 μl of deoxynucleoside triphosphates (25 mM), 3 μl of $MgCl_2$ (25 mM), 0.1 μl of *Taq* polymerase (250 U) (Applied Biosystems, Foster City, Calif.), 2 μl of DNA template (10 to 100 ng), and 1 μl of each primer (100 to 200 ng each). The reaction cycle parameters included an initial denaturation step consisting of 5 min at 95°C, followed by a set of seven touchdown PCR cycles (7) (30 s of denaturation at 94°C, 30 s of annealing at 62, 60, 59, 58, 57, 56, and 55°C, and then 30 s of elongation at 72°C) and then 30 cycles of denaturation at 94°C, annealing for 30 s at 54°C, and elongation for 30 s at 72°C, with a final extension step consisting of 5 min at 72°C. PCR products were examined to determine sizes and yields by using a 0.8% (wt/vol) agarose gel and TAE buffer (20 mM Tris-HCl, 10 mM sodium acetate, 0.5 mM Na_2EDTA; pH 8.0) (21).

DGGE. Analysis of PCR products by DGGE was performed as described by Tal et al. (31) by using the D-Code universal mutation detection system (Bio-Rad, Hercules, Calif.). Products from triplicate PCRs were combined and applied directly to 10% (wt/vol) polyacrylamide gels that contained a 35 to 50% gradient of denaturant (100 ml of 100% denaturant contained 42 g of urea, 40 ml of deionized formamide, 25 ml of a 40% acrylamide-bisacrylamide solution [37.5:1], and 1 ml of 50× TAE buffer). Electrophoresis was performed for 5 h at 60°C in TAE buffer at 200 V (constant voltage). After electrophoresis, nucleic acids were stained with SYBR Green II (1:10,000 dilution; Molecular Bio-Probes, Eugene, Oreg.), and the staining pattern was immediately recorded by using a fluoroimager (Molecular Dynamics, Sunnyvale, Calif.). DNA fragments were isolated by staining gels with ethidium bromide (0.5 μg/ml) and were visualized by using a UV transilluminator. Gel sections containing fragments of interest were excised, placed in 50 mM Tris-HCl (pH 8.0)–1 mM Na_2EDTA, and incubated at 4°C overnight. The eluted DNA was used as a template for the PCR, as described above, and was checked for purity by DGGE.

Cloning, sequencing, and database analysis. PCR-amplified DNA and DNA fragments obtained from DGGE gel slices were introduced into a pCR2A vector and transformed into *Escherichia coli* by using a TOPO TA cloning kit according to the manufacturer's instructions (Invitrogen, Carlsbad, Calif.). The presence of cloned inserts was verified by PCR amplification, and sequence analysis was carried out by using an ABI 373 automated sequencer (PE Applied Biosystems, Foster City, Calif.). DNA sequences were analyzed by using FASTA (18) and a BLAST search of nucleic acid databases (1). Partial 16S rRNA gene sequences were manually compiled and aligned by using the PHYDIT software (4). Evolutionary trees were generated by using the neighbor-joining (19) algorithm in the PHYLIP software (10). Evolutionary distance matrices for the neighbor-joining and Fitch-Margoliash methods were generated as described by Jukes and Cantor (13). The robustness of the inferred tree topologies was evaluated after 1,000 bootstrap resamplings of neighbor-joining data.

FISH analysis. One gram of sediment was suspended in 5 ml of phosphate-buffered saline (PBS) (20) (pH 8.0) and vortexed gently for 3 min, followed by centrifugation at 1,000 × *g* for 30 s. The supernatant fraction was centrifuged at 14,000 × *g* for 10 min and resuspended in PBS. Cells were fixed with 4% (vol/vol) paraformaldehyde in PBS for 1 h at room temperature, followed by two washes with PBS. Fixed cells were suspended in a 50% (vol/vol) PBS–ethanol solution and stored at −20°C. Aliquots (10 μl) of the fixed cell stock were used for fluorescence in situ hybridization (FISH) analysis as described previously (27). Texas Red-labeled anammox-specific probe Amx820 (Sigma Genosys) was used for detection of anammox bacteria, and counterstaining was done by using 4,6-diamidino-2-phenylindole (DAPI). Image acquisition was done with an Olympus AX-60 microscope equipped with the appropriate filter set and a black and white digital camera. Approximate anammox bacterial densities were determined by comparing the number of cells stained with the anammox-specific Amx820 probe to the number of bacteria stained with DAPI, and the average number was obtained by analyzing five different slides.

Nucleotide sequence accession numbers. Sequences of the partial 16S rRNA genes of the predominant restriction fragment length polymorphism types have been deposited in the GenBank database under accession numbers AY266449, AY360085, AY266450, and AY360082.

RESULTS

Evaluation of anammox activity in Baltimore Inner Harbor sediment. Inner Harbor sediment was examined to determine its ability to convert [^{15}N]ammonia to N_2 gas in the presence of nitrite. Incubation of sediment with either [^{15}N]ammonia, ammonia, or nitrite did not result in the production of detectable concentrations of $^{29}N_2$ or $^{30}N_2$ in bottle headspace gases (data not shown). Addition of both [^{15}N]ammonia and nitrite, however, resulted in a decrease in their concentrations over time (Fig. 1C) that coincided with an increase in the concentrations of $^{29}N_2$ and measurable levels of $^{30}N_2$ (Fig. 1A and B). The $^{29}N_2$ concentration reached a peak of 2.1 μM after 21 days of

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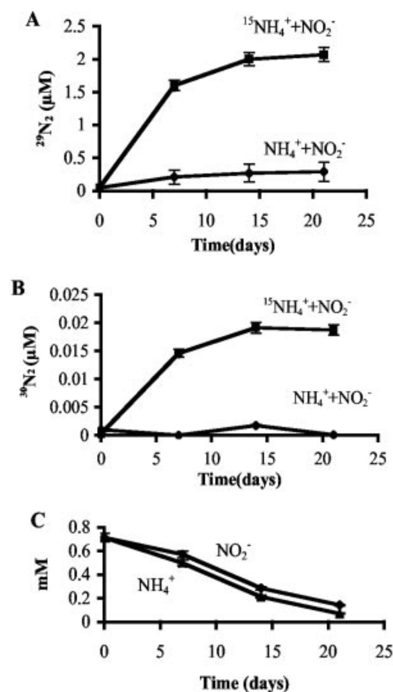

FIG. 1. Anammox activity of Baltimore Inner Harbor sediment measured by ^{15}N tracer studies. Inner Harbor sediment was incubated under anoxic conditions in the presence of ammonia and nitrite as described in Materials and Methods. $^{29}N_2$ (A) or $^{30}N_2$ (B) concentrations in bottle headspace were measured with sediment incubated either with [^{15}N]ammonia and nitrite or with unlabeled ammonia and nitrite. (C) Total ammonia and nitrite concentrations during incubation.

incubation (Fig. 1A), with most of the $^{29}N_2$ production occurring within the first 100 h of incubation at a rate of approximately 0.2 μM $^{29}N_2$ produced day^{-1} g of sediment^{-1}. The $^{30}N_2$ concentrations, on the other hand, increased with time, and the pattern of accumulation was the same as the patterns for the $^{29}N_2$ isotope, although the final concentration was only 0.018 μM and the initial rate was approximately 0.002 μM $^{30}N_2$ produced day^{-1} g of sediment^{-1} (Fig. 1B). These results suggested that anammox activity was likely associated with the microbial community of the sediment, which includes members of the *Planctomycetales*.

Isolation and analysis of anammox-specific 16S rRNA genes from Inner Harbor sediment. Identification of anammox-related 16S rRNA gene sequences involved a sequential PCR approach that enhanced our ability to amplify these sequences from the sediment bacterial population. Initially, DNA from sediment material was extracted and subjected to PCR amplification by using the *Planctomycetales*-specific Pla46 primer and the general bacterial 1392r oligonucleotide primer. Under stringent conditions, amplification generated a library of partial *Planctomycetales* 16S rRNA genes that was then cloned into *E. coli*. Of several hundred transformants isolated, we chose 50 that harbored the cloned Pla46-1392r insert. Screening these clones with primer Pla46 and anammox-specific primer Amx820 resulted in identification of two clones, A6 and A62, which yielded an approximately 750-bp PCR product. An additional two clones, clones B4 and C6, generated an approximately 1,200-bp product when primer Pla46 and anammox-specific primer Amx1240 were used for amplification.

DNA sequence analysis of the four cloned Pla46-1392r segments (GenBank accession numbers for these sequences are shown in the legend to Fig. 2) revealed differences in the anammox signature Amx820 and Amx1240 primer regions at several positions compared to the same regions in *K. stuttgartiensis* and *B. anammoxidans* (Fig. 2), and FASTA analysis showed that the levels of sequence similarity to both *K. stuttgartiensis* and *B. anammoxidans* for all of the clones ranged from 79 to 84%, compared to 91% similarity between *K. stuttgartiensis* and *B. anammoxidans*. We constructed a phylogenetic tree of the 16S rRNA gene sequences that included representative *Planctomycetales*, the known anammox bacteria, and the Baltimore Inner Harbor clones (Fig. 3). Of the four clones, clone A62 was most closely related to the anammox cluster, although it was outside the groups containing *B. anammoxidans* and *K. stuttgartiensis*. Clones A6, B4, and C6, on the other hand, clustered together as a subgroup that was separate from but most closely related to the anammox group.

DGGE analysis. Establishing that the four anammox clones could be detected in the Inner Harbor sediment was accomplished by DGGE analysis. Electrophoresis was performed with PCR products generated with universal bacterial primers 1055 and 1392rGC, and DNA was extracted from a sediment sample before and after enrichment in anammox-specific media (Fig. 4). Bands that comigrated with PCR-amplified DNA from clones A62, A6, B4, and C6 were detected in the anammox-enriched sample (Fig. 4) and were excised from the gel. The samples were then subjected to an additional PCR amplification by using the 1055-1392rGC primer set, and they were cloned and sequenced (see Materials and Methods). Our results showed that the DNA sequences of all four isolated fragments were identical to the sequences of the comigrating anammox clones (data not shown), indicating that these anammox 16S rRNA gene sequences were initially present in the sediment material and were enhanced under anammox-enriching conditions. In addition to the four anammox sequences, we

A

Amx 820	GGGCACTAAGTAGAGGGGTTTT
B. anammoxidans	GGGCACTAAGTAGAGGGGTTTT
K. stuttgartiensis	GGGCACTAAGTAGAGGGGTTTT
Clone A6	GGGCACTAAGCAGTGGGGTTTT
Clone A62	GGGCACTAGGTAGAGGGAGTTC
Clone B4	GAGCACTAGGTAGTGGCAAGTC
Clone C6	GAGCACTAGGTAGCCGCCGGAG

B

Amx 1240	GGTTGGTACAAAGGGATGCTAAA
B. anammoxidans	GGTTGGTACAAAGGGATGCTAAA
K. stuttgartiensis	GGTCGGTACAAAGGGATGCTAAG
Clone A6	GGCACGTACAAAGGGAAGCGAAC
Clone A62	GGCGGATACAAAGGGAAGCAAGA
Clone B4	GGCCGGTACAAAGGGTTGCCAAC
Clone C6	GGCCGGTACAAAGGGTCGCGAAC

FIG. 2. Comparison of the Baltimore Inner Harbor isolate sequences with anammox signature regions. Clone sequences in regions around 770 and 1,200 bp from the 5′ (Pla46) end are compared to the Amx820 (A) and Amx1240 (B) oligonucleotide primers and *B. anammoxidans* (accession number AF375994) and *K. stuttgartiensis* (accession number AF375994) sequences. Bases that differ from bases in the primer sequences are underlined. The GenBank accession numbers for clones A6, A62, B4, and C6 are AY266449, AY360085, AY266450, and AY360082, respectively.

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Thermotoga maritima
Nitrospira marina
Nitrobacter winogradskyi
Nitrosomonas marina
Clone A62 (AY360085)
Kuenenia stuttgartiensis (AF375995)
KOLL2a (AJ250882)
AF202663
AF202655
Anammox clone 14 (AJ131819)
Brocadia anammoxidans (AF375994)
Scalindua wagneri (AY254882)
Scalindua brodae (AY254883)
Scalindua sorokinii (AY257181)
Clone B4 (AY266450)
Clone C6 (AY360082)
Clone A6 (AY266449)
Gemmata obscuri
Pirellula limnophilus
Pirellula maris
Pirellula brasiliensis
Pirellula 678
Pirellula marina
Pirellula schlesner
Isosphaera pallida
Anammox *Planctomycetales*
Planctomycetales
86 99 72 100 98 96 100 100 80 100 100 100 96 100 93 98 96 62 97 84 100
0.1

FIG. 3. Neighbor-joining phylogenetic tree generated from analysis of 1,120 bp of 16S rRNA gene sequences, showing the relationship of the 16S rRNA gene sequences from the Baltimore Inner Harbor isolates to *Planctomycetales*. The numbers at the nodes are percentages indicating the levels of bootstrap support based on a neighbor-joining analysis of 1,000 resampled data sets (only values greater than 50% are shown). *Thermatoga maritima* was used as an outgroup.

also identified a fragment that had sequence similarity with the 16S rRNA gene from *Nitrosomonas* sp., which exhibited high band intensity in the DNA from the anammox-enriched sediment (Fig. 4). *Nitrosomonas* sp. is frequently associated with anammox bacteria in the communities of enriched cultures (14), and our results demonstrated that the Inner Harbor sediment has both anammox activity and potential nitrifying ability.

FISH analysis. The microbial population of the Inner Harbor sediment was examined by in situ hybridization studies by using the Amx820 probe fluorescently labeled with Texas Red. As Fig. 5 shows, several DAPI-stained bacteria (Fig. 5A) were labeled by the anammox-specific 16S rRNA gene probe (Fig. 5B); counts of the Amx820-stained cells yielded an anammox bacterial density of approximately 300 $\pm$ 100 cells ml^{-1} (0.4 to 0.6% of the DAPI-stained cells). This density approaches the range reported by Kuypers et al. (15) for the Black Sea water column and provides further evidence that anammox-related bacteria may be found in the Inner Harbor sediment consortium.

DISCUSSION

Our detection of anammox activity in Baltimore Inner Harbor sediment and our identification of anammox-related bacteria both microscopically and by isolation of new 16S rRNA

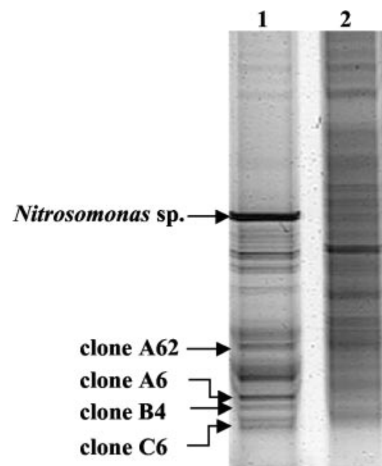

FIG. 4. DGGE analysis of PCR-amplified 16S rRNA gene fragments. DNA was extracted from a sediment sample before (lane 2) and after (lane 1) enrichment in the presence of ammonia and nitrite and amplification with the universal 1055 and 1392rGC primers, and electrophoresis was performed as described in Materials and Methods. The positions of fragments that comigrated with Inner Harbor anammox clones that were amplified with the same universal primer set and a fragment having similarity to *Nitrosomonas* sp. are indicated.

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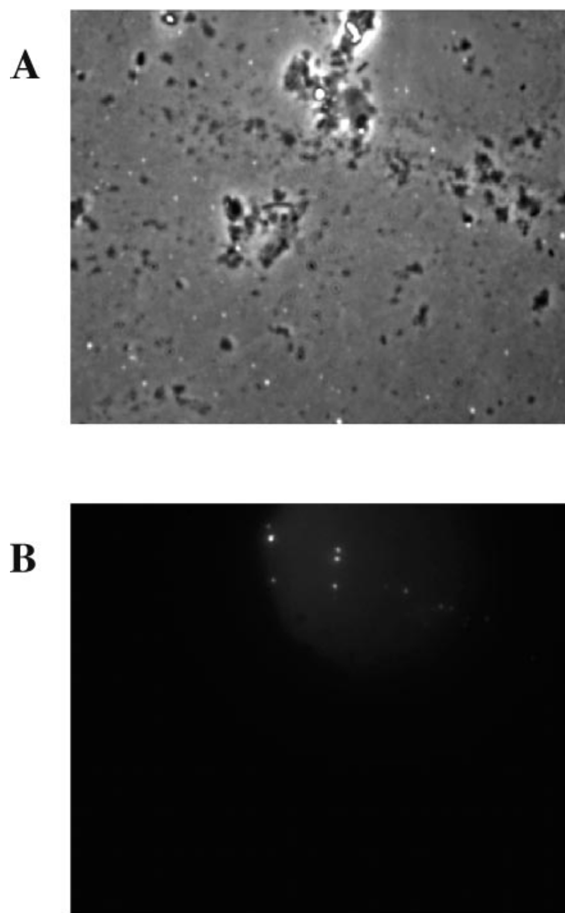

FIG. 5. Detection of anammox bacteria in Inner Harbor sediment by FISH analysis. (A) Universal bacterial stain with DAPI. (B) Same field image visualized with Texas Red filters after hybridization with Texas Red-labeled probe Amx820. Magnification, ×1,000.

gene sequences provide the first report of the occurrence of this important process and related bacteria in the Chesapeake Bay estuarine basin. To our knowledge, this is also the first demonstration of both anammox activity and associated 16S rRNA genes in sediment. In previous sediment studies workers measured anammox activity, but sequences of anammox bacteria were not identified (33). In addition, aside from the characterization of anammox bacteria and their activity in wastewater treatment systems, studies in which both anammox activity and related sequences have been described have focused on samples from anoxic water column environments (6, 15). Thus, taken together, our results provide further evidence for the global distribution of anammox-capable *Planctomycetales* species.

Using anammox-specific Amx820 and Amx1240 oligonucleotide primer sets to screen clones from a *Planctomycetales* 16S rRNA gene library, we identified four clones from sediment material that exhibited low overall levels of sequence similarity to the prototypical *K. stuttgartiensis* and *B. anammoxidans* anammox bacterial isolates. While the new 16S rRNA gene sequences varied considerably in the Amx820 and Amx1240 signature regions, the fact that they could be amplified by using the highly conserved anammox-specific Amx820 or Amx1240 sequences confirmed that these clones are part of the anammox-capable *Planctomycetales* group of bacteria. The sequence variations may be useful for the development of more inclusive anammox-specific primers, as it is clear from other studies that populations of anammox-performing bacteria are not detected by currently used oligonucleotide primers (33).

Our phylogenetic analysis indicated that the four new clones clustered with the anammox group. Three of the new anammox 16S rRNA gene sequences (A6, B4, and C6) form a cohesive new branch of the anammox group, and the remaining clone (A62) appears to branch separately from this group. However, the bootstrap value for the A62 node is low (72%) and may change with addition of other sequences to this group. Additional analyses with isolated individuals are necessary to determine the detailed phylogenetic relationships of the members of this diverse group. Nevertheless, genus level diversity of anammox *Planctomycetales* has been demonstrated in other studies (23), and we confirmed this notion and extended it to other environments.

Incubations of Inner Harbor sediment with [^{15}N]ammonia and nitrite resulted in predominately accumulation of $^{29}N_2$ in the gas phase, which is consistent with studies that showed that the anammox reaction involved the pairing of nitrogen atoms from ammonia and nitrite at a 1:1 ratio (33). The accumulation of $^{30}N_2$ during the incubation was 2 orders of magnitude lower than that of $^{29}N_2$, suggesting that there was a random pairing of nitrogen atoms that was not exclusively generated by the anammox process (6, 33). Interestingly, a band corresponding to *Nitrosomonas* sp. was identified in the DGGE analysis of the enriched Inner Harbor sediment (Fig. 4), which is consistent with studies demonstrating that anammox and nitrifying bacteria can share the same aerobic or even anaerobic environments (25). Furthermore, attempts to isolate anammox bacteria as pure cultures frequently result in mixed communities of anammox and *Nitrosomonas* sp. (14). The role of *Nitrosomonas* sp. in either production of $^{30}N_2$ or the anammox process itself has not been determined.

Whether the anammox activity that we measured may be directly attributed to the anammox *Planctomycetales* sequences obtained from the sediment or is due to other uncharacterized bacteria cannot be determined from our studies, and determining the relationship between activity and sequence requires the isolation and characterization of these bacteria. However, the fact that all four 16S rRNA gene sequences could be detected in the DGGE profile after enrichment in the presence of anammox-specific media makes it likely that these bacteria play some role in sediment anammox activity and may be important in the overall nitrogen budget of the Chesapeake Bay.

The ^{15}N tracer studies indicated that within the first 100 h of incubation with [^{15}N]ammonia and nitrite, Inner Harbor sediment had the capacity to produce $^{29}N_2$ at a rate of approximately 0.2 μM day^{-1} g of sediment^{-1} (Fig. 1A). Evaluating the significance of this production rate requires estimation of the denitrification activity in Inner Harbor sediment. The relative roles of the two processes in the bay's nitrogen budget remain to be determined, although it has been shown that anammox activity in similar marine sediments could account for between 24 and 67% of total nitrogen gas formation (33).

A prominent characteristic of the Baltimore Inner Harbor

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sediment is its high level of organic load and pollutants, such as polychlorinated benzenes and heavy metals (2). While the relationship between organic load and anammox activity is not clear, a recent study concluded that high organic loading favors the anammox process due to the formation of a thick biofilm that enhances the anoxic zone, establishing and supporting anammox activity (31). However, it has also been shown that no significant change in anammox activity occurs after the addition of organic matter (33). Understanding the role of anammox in the bay's nitrogen cycle and determining whether the process is limited to urban polluted sites or is controlled by the degree of nutrient wash-out into the bay will be the focus of future studies.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Baker, J., R. Mason, J. Cornwell, J. Ashley, J. Halka, and J. Hill.** 1997. Spatial mapping of sedimentary contaminants in the Baltimore Harbor/Patapsco River/Back River system. Maryland Department of the Environment. University of Maryland Center for Environmental Studies, Chesapeake Bay Laboratory, Solomons, Md.
3. **Boesch, D. F., R. B. Brinsfield, and R. E. Magnien.** 2001. Chesapeake Bay eutrophication: scientific understanding, ecosystem restoration, and challenges for agriculture. J. Environ. Qual. **30:**303–320.
4. **Chun, J.** 1995. Computer-assisted classification and identification of actinomycetes. Ph.D. dissertation. University of Newcastle upon Tyne, Newcastle upon Tyne, United Kingdom.
5. **Cutter, L., K. R. Sowers, and H. D. May.** 1998. Microbial dechlorination of 2,3,5,6-tetrachlorobiphenyl under anaerobic conditions in the absence of soil or sediment. Appl. Environ. Microbiol. **64:**2966–2969.
6. **Dalsgaard, T., D. E. Canfield, J. Petersen, B. Thamdrup, and J. Acuna-Gonzalez.** 2003. N_2 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica. Nature **422:**606–608.
7. **Don, R. H., P. T. Cox, B. J. Wainwright, K. Baker, and J. S. Mattick.** 1991. 'Touchdown' PCR to circumvent spurious priming during gene amplification. Nucleic Acids Res. **19:**4008.
8. **Egli, K., F. Bosshard, C. Werlen, P. Lais, H. Siegrist, A. J. Zehnder, and J. R. Van der Meer.** 2003. Microbial composition and structure of a rotating biological contactor biofilm treating ammonium-rich wastewater without organic carbon. Microb. Ecol. **45:**419–432.
9. **Egli, K., U. Fanger, P. J. Alvarez, H. Siegrist, J. R. van der Meer, and A. J. Zehnder.** 2001. Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch. Microbiol. **175:**198–207.
10. **Felsenstein, J.** 1989. PHYLIP—Phylogeny Inference Package (version 3.2). Cladistics **5:**164–166.
11. **Ferris, M. J., G. Muyzer, and D. M. Ward.** 1996. Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Appl. Environ. Microbiol. **62:**340–346.
12. **Holoman, T. R., M. A. Elberson, L. A. Cutter, H. D. May, and K. R. Sowers.** 1998. Characterization of a defined 2,3,5,6-tetrachlorobiphenyl-*ortho*-dechlorinating microbial community by comparative sequence analysis of genes coding for 16S rRNA. Appl. Environ. Microbiol. **64:**3359–3367.
13. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism. Academic Press, New York, N.Y.
14. **Kuenen, J. G., and M. S. M. Jetten.** 2001. Extraordinary anaerobic ammonium-oxidizing bacteria. ASM News **67:**456–463.
15. **Kuypers, M. M., A. O. Sliekers, G. Lavik, M. Schmid, B. B. Jorgensen, J. G. Kuenen, J. S. Sinninghe Damste, M. Strous, and M. S. Jetten.** 2003. Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. Nature **422:**608–611.
16. **Lindsay, M. R., R. I. Webb, M. Strous, M. S. Jetten, M. K. Butler, R. J. Forde, and J. A. Fuerst.** 2001. Cell compartmentalisation in planctomycetes: novel types of structural organisation for the bacterial cell. Arch. Microbiol. **175:**413–429.
17. **Neef, A., R. Amann, H. Schlesner, and K. H. Schleifer.** 1998. Monitoring a widespread bacterial group: *in situ* detection of planctomycetes with 16S rRNA-targeted probes. Microbiology **144:**3257–3266.
18. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
19. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
21. **Schalk, J., H. Oustad, J. G. Kuenen, and M. S. Jetten.** 1998. The anaerobic oxidation of hydrazine: a novel reaction in microbial nitrogen metabolism. FEMS Microbiol. Lett. **158:**61–67.
22. **Scheiner, D.** 1976. Determinations of ammonia and Kjeldahl nitrogen by the indophenol method. Water Res. **10:**31–36.
23. **Schmid, M., U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. Jetten, J. W. Metzger, K. H. Schleifer, and M. Wagner.** 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Syst. Appl. Microbiol. **23:**93–106.
24. **Schmid, M., K. Walsh, R. Webb, W. I. Rijpstra, K. van de Pas-Schoonen, M. J. Verbruggen, T. Hill, B. Moffett, J. Fuerst, S. Schouten, J. S. Damste, J. Harris, P. Shaw, M. Jetten, and M. Strous.** 2003. Candidatus "*Scalindua brodae*" sp. nov., Candidatus "*Scalindua wagneri*" sp. nov., two new species of anaerobic ammonium oxidizing bacteria. Syst. Appl. Microbiol. **26:**529–538.
25. **Schmidt, I., O. Sliekers, M. Schmid, I. Cirpus, M. Strous, E. Bock, J. G. Kuenen, and M. S. Jetten.** 2002. Aerobic and anaerobic ammonia oxidizing bacteria—competitors or natural partners? FEMS Microbiol. Ecol. **39:**175–180.
26. **Sinninghe Damste, J. S., M. Strous, W. I. Rijpstra, E. C. Hopmans, J. A. Geenevasen, A. C. van Duin, L. A. van Niftrik, and M. S. Jetten.** 2002. Linearly concatenated cyclobutane lipids form a dense bacterial membrane. Nature **419:**708–712.
27. **Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K. H. Schleifer.** 1997. Phylogenetic analysis and in situ identification of bacteria in activated sludge. Appl. Environ. Microbiol. **63:**2884–2896.
28. **Strickland, J. D., and T. R. Parsons.** 1968. A practical handbook of seawater analysis. Fisheries Research Board of Canada, Ottawa, Canada.
29. **Strous, M.** 2000. Microbiology of anaerobic ammonium oxidation. Ph.D. thesis. Technical University of Delft, Delft, The Netherlands.
30. **Strous, M., J. A. Fuerst, E. H. Kramer, S. Logemann, G. Muyzer, K. T. van de Pas-Schoonen, R. Webb, J. G. Kuenen, and M. S. Jetten.** 1999. Missing lithotroph identified as new planctomycete. Nature **400:**446–449.
31. **Tal, Y., J. E. Watts, S. B. Schreier, K. R. Sowers, and H. J. Schreier.** 2003. Characterization of the microbial community and nitrogen transformation processes associated with moving bed bioreactors in closed recircualted mariculture systems. Aquaculture **215:**187–202.
32. **Thamdrup, B., and T. Dalsgaard.** 2000. The fate of ammonium in anoxic manganese oxide rich marine sediment. Geochim. Cosmochim. Acta **64:**4157–4164.
33. **Thamdrup, B., and T. Dalsgaard.** 2002. Production of N_2 through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Appl. Environ. Microbiol. **68:**1312–1318.
34. **Ward, B. B.** 2003. Significance of anaerobic ammonium oxidation in the ocean. Trends Microbiol. **11:**408–410.

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