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Research Article

Combine Cultivation and Independent Molecular Approach to Identify Ammonia Oxidizing Bacteria in Industrial Waste Water Treatment

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Abstract

To identify the confirmed presence of ammonia oxidizing bacteria, culture enrichments and culture-independent molecular methods were employed. Biomass from nitrifying systems were seeded and enriched for ammonia oxidizing bacteria under various conditions of ammonia concentration in bioreactors. Surveys of cloned rRNA genes from the enrichments revealed four major strains of AOB which were phylogenetically related to the *Nitrosomonas marina* cluster, the *Nitrosospira* cluster, or the *Nitrosomonas europaea-Nitrosococcus mobilis* cluster of the β subdivision of the class *Proteobacteria*. In enrichment ammonia concentration in the reactors determined which AOB strain dominated. PCR primers sets and Oligonucleotide probes were developed and used for the four Ammonia oxidizing bacterial strains to confirm the presence in the enrichments. Enriched ammonia oxidizing strains were added to waste water to determine their ability to accelerate the establishment of ammonia oxidation. *Nitrosomonas marina*-like AOB strain was most efficient at accelerating ammonia oxidation. Furthermore, if the *Nitrosomonas marina*-like AOB strain was present in the original enrichment, only the *Nitrosomonas marina*-like AOB strain was present in aquaria after nitrification was established. *Nitrosomonas marina*-like AOB were 2% or less of the cells detected by fluorescence in situ hybridization analysis in aquaria in which nitrification was well established.

Keywords: Ammonia Oxidizing Bacteria; Industrial Waste Water; Nitrosomonas; Enrichment; PCR

Introduction

Rapid industrialization has necessitated the manufacture and use of different chemicals in day to day life [1]. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide [2]. Pollution due to textile industry effluent has increased during recent years. Moreover, it is very difficult to treat textile industry effluents because of their high BOD, COD, heat, color, pH and the presence of metal ions [3]. Ammonia in aquatic environments can be toxic to fish and other aquatic life and contributes to eutrophication of water bodies [4]. Accordingly, removal of ammonia in wastewater is one of the primary tasks of the modern wastewater treatment process. A widely used method to remove ammonia in Waste Water Treatment Plant (WWTP) is biological nitrification by which ammonia is oxidized to nitrite by Ammonia Oxidizing Bacteria (AOB) and then nitrite is subsequently oxidized to nitrate by Nitrite Oxidizing Bacteria (NOB). Although activated sludge is a common process for wastewater treatment, nitrification failure unfortunately occurs frequently in many WWTPs [5,6], since nitrifiers, particularly AOB, grow very slowly, and they are highly sensitive to several environmental factors, including temperature, pH, Dissolved Oxygen (DO), and a wide variety of chemical inhibitors [7,8]. Therefore, a better understanding of the microbial ecology of AOB in WWTPs could potentially improve the nitrification stability [9]. Culture-dependent methods are biased by the selection of species which obviously do not represent the real dominance structure, and hence give a poor understanding of AOB community structure [9]. Recent studies of many environments have demonstrated a large amount of diversity among ammonia-oxidizing bacteria (AOB) [10-14]. AOB are responsible for the first step in nitrification, conversion of ammonia to nitrite, and are members of the β subdivision of the class *Proteobacteria* except for the marine genus *Nitrosococcus*, which belongs to the β subdivision [15]. Historically, *Nitrosomonas europaea* has generally been believed to be the bacterium responsible for ammonia oxidation [16]. However, the application of cultivation-independent molecular techniques, including rRNA gene surveys [17], Fluorescent In Situ Hybridization (FISH) [18], and Denaturing Gradient Gel Electrophoresis (DGGE) [19], has removed biases associated with cultivation, and additional AOB have been identified from a number of environments, including soils [20], sand dunes [21], biofilms [22], fluidized bed reactors [23,13], lakes [24], wastewater [11], and seawater [25]. The aim of this study was to combine cultivation-independent molecular techniques and cultivation methods to identify AOB responsible for ammonia oxidation in industrial waste water systems. Several enrichments of AOB were established from material collected from actively nitrifying freshwater systems. A range of molecular techniques were applied to identify the confirmed presence of putative AOB in the enrichments.

Materials and Methods

Biomass Source

A biomass with a mixed culture nitrifying bacteria was obtained

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Table 1: Source and age of the nitrifying biomasses.

BioFarm I6	BioFarm sump and BioWheel biomass	3.5	35-65
BCF	BioFarm sump and BioWheel biomass	2	15-May
BC5(2)	BioFarm sump and BioWheel biomass	2	20-May
R3	Biomass from an aquarium cultured for 335 days	9 month in reactor	20-May
R7	BC5 biomass	9 month in reactor	10-May
R5	Water filter from ammonia feed stock solution reservior	9 month in reactor	40-70

from the activated sludge of common effluent treatment plant, India. The activated sludge was filtered to remove waste and washed repeatedly to remove internal nitrogen components. The sludge was then dewatered and kept in a cold room (4° C) for future use.

Experimental Set-up

The enrichment of high ammonia oxidizing bacteria was conducted in a laboratory scale bioreactor with a working volume of 5 Ltr. The bioreactors were kept in darkened cabinets at 26°C. The influent comprised a simple autotrophic medium contains potassium phosphate (0.5 mg/liter) & ammonium chloride. The ammonia N concentration was kept in the range of 5 to 10 mg/liter for the low-concentration ammonia reactors and 40 to 60 mg/liter for the high-concentration ammonia reactors (Table 1). Bioreactors were monitored daily and maintained at their predefined ammonia N concentrations by feeding the autotrophic media when required. Nitrate, nitrite and ammonia concentrations were routinely monitored by flow injection analysis and ion chromatography [26,27]. The pH of the bacterial suspensions was kept at or above a pH of 8.0 through the addition of sodium bicarbonate. For analysis of the enriched bioreactor biomass, a sample was eliminated via the sampling port after the bioreactor's internal surfaces had been scrubbed and the biomass was evenly resuspended.

Extraction and purification of DNA

One gram samples were washed (twice) with 2 volumes of 120 mM sodium phosphate buffer (pH 8.0) in order to elute naked DNA [28]. After centrifugation the pellets were resuspended in 500 ml of sodium phosphate buffer (pH 8.0) to which 0.5 g of glass beads and 500 ml of phenol-chloroform-isoamyl alcohol (25:24:1 [vol/vol/vol]) were added. Lysis of cells in soil samples was done by bead beating for 1 min in a Microdis membrator U set to 2,000 rpm. Sediment samples were treated in a similar manner except that a vortex mixer was employed for two 1-min periods to lyse cells. After centrifugation the aqueous supernatant was removed and the pellet was re-extracted as described above. Pooled supernatants from samples were concentrated and repeatedly dialyzed with Tris-EDTA buffer (pH 8.2) in a Microcon 100 spin dialysis unit until no brown pigment appeared in the eluate. The retentate was further purified by gel electrophoresis through a 1% (wt/vol) low-melting-point agarose gel. A band containing DNA of an estimated molecular mass greater than 8 kb was excised and trimmed of excess agarose, and the DNA was purified by use of Qiaex resin. Samples were treated similarly, except that the repeated dialysis step was omitted and DNA extraction from agarose gels was performed with a Spin bind DNA Recovery System.

PCR amplification of 16S rDNA, cloning, and sequencing

Approximately 20 ng of DNA was used in a 50-ml PCR reaction mixture. The primers bAMOf and bAMOr, designed to selectively amplify approximately 1.1 kb of 16S rDNA from b-subgroup ammonia oxidizers and their close relatives, have been described previously [28,29]. Two units of *Taq* DNA polymerase was used in PCR reaction mixtures with an annealing temperature of 55 °C in a buffer supplied by the manufacturer. Other PCR conditions were as described previously [24]. PCR products from five reactions were pooled to minimize possible bias due to random events within individual PCR reactions [5]. The PCR band was purified for cloning by gel electrophoresis followed by extraction of the excised band with Qiaex resin. Ligations into the pGEM T vector followed the manufacturer's protocols. The ligations were transformed into XL1-Blue MRF Kan super competent *Escherichia coli* and plated on Luria-Bertani agar supplemented with IPTG (isopropyl-b-D-thiogalactopyranoside [1 mM]), X-Gal (5-bromo-4-chloro- 3-indolyl-b-D-galactopyranoside [40 mg ml21]) and the antibiotics kanamycin, ampicillin, and methicillin according to manufacturer's instructions. White colonies were grown overnight in Luria-Bertani broth (5 ml) containing ampicillin (50 mg ml21) at 37 °C with shaking. Plasmids were purified by using the Promega Wizard Mini-Prep system. Approximately 200 plasmids were analyzed from each soil clone library by a single dideoxynucleotide (ddA; Ttracking) - sequencing method and primer 519r [5,30-32] in order to identify unique clones. These were then partially sequenced for about 300 bases with primer 519r. Approximately 40 clones were randomly chosen from the sediment libraries for partial sequencing. Fifteen clones chosen to be representative of the different groups revealed by partial sequence analysis were fully sequenced for the entire 1.1-kb insert with the SP6 and T7 plasmid primers plus internal primers. Manual sequencing used Sequenase and 10% (vol/vol) dimethyl sulfoxide in all reaction mixtures. Double stranded PCR products from enrichment cultures were precipitated and sequenced directly [8,23,33].

Real Time Polymerase Chain Reaction

qPCR was performed using an iCycler IQ System (Bio-Rad, Hercules, USA), two replicates for each sample. For AOA, the PCR was performed in a total volume of 30 µl containing 15 µl of FailSafe™ PCR Premix F, 5 µl of DNA template, 0.1 µM of each primer, 1.5 U of AmpliTaq® DNA polymerase, 15 mM MgCl2, and 0.5× SYBR® Green I (Invitrogen, Eugene, USA). For AOB, the PCR was performed in a total volume of 30 µl containing 15 µl of iQ™ SYBR® Green Super Mix (Bio-Rad, Hercules, USA), 5 µl of DNA template, and 0.1 µM of each primer. The real-time PCR thermocycling steps were set as follows: 95°C for 4 min and 45 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s. Cycling was completed by a final elongation step at 72°C for 10 min. The negative control containing no DNA was subjected to the same procedure to exclude or detect any possible contamination. After real-time PCR assay, the specificity of amplification was verified by generation of melting curves and agarose gel electrophoresis

Table 2: The PCR sequencing primers and FISH oligonucleotides for general and specific detection of AOB.

Primer or probe	Sequence (5'-3')	Target site	Annealing temp (°C)	NaCl concn (mM)	% Formamide	Specificity
CTO189f	GGA GRA AAG YAG GGG ATC G	189-207	57			β proteobacterial AOB
NITROSO4Er	CAC TCT AGC YTT GTA GTT TC	639-658	57			β proteobacterial AOB
NSMR71f	CGG AAC GTA TCC AGA AGA	126-143	54			*Nitrosonionas marina*-like AOB
NSMR74r	ATC TCT AGA AAA TTC GCT	1,000-1,017	54			*Nitrosomonas marina*-like AOB
NSMR32f	ATC GGA ACG TAT CTT CG	125-141	56			*Nitrosospim* tennis-like AOB
NSMR33r	CCA CCT CTC RGC GGG C	1,006-1,021	56			*Nitrosospgra tennis*-like AOB
NSMR52f	TCA GAA AGA AAG AAT CAT G	443-461	56			*Nitrosomonas europaea* -like AOB
NSMR53r	GTC TCC AYT AGA TTC CAA G	999-1,017	56			*Nitrosomonas europaece*-like AOB
NMOB1f	GTT GGG AAG AAA CGA TTR CA	442-461	56			*Nitrosococcus mobilis*-like AOB
NMOB1r	CAC TTT TAT GTC TCC GTA AAA	1,006-1,026	56			*Nitrosococcus mobilis*-like AOB
EUB338	GCT GCC TCC CGT AGG AGT	338-355		225	20	Bacteria
NITROSO4E	CAC TCT AGC YTT GTA GTT TC	639-658		225	20[a]	β proteobacterial AOB
Nso190	CGA TCC CCT GCT TTT CTC C	190-209		20	55	β proteobacterial AOB
NSMR76	CCC CCC TCT TCT GGA TAC	132-149		225	20	*Nitrosomonas marina*-like AOB
NSMR34	TCC CCC ACT CGA AGA TAC G	131-149		225	20	*Nitrosospim* tennis-like AOB
S-G-Ntspa-0685-a-A-22	CAC CGG GAA TTC CGC GCT CCT C	664-685		225	20[b]	*Nitrospira-like* NOB

[a]Established empirically in this study, probe originally used for slot blots [12].
[b]Established empirically in this study, probe originally used for slot blots [13].

Table 3: Number of clones screened and sequenced for each clone library and numbers of clones for each AOB strain from this study found in the libraries.

Group	No. of clones for:						
	Bio-Farm16	BC5	BC5 (2)	R3	R7	R5	R7 Post BA
Clones screened[a]	54	76	104	36	185	105	83
Clones fully sequenced	20	21	2	12	18	15	4
Clones partially sequenced	34	47	86	6	110	21	44
β proteobacteria AOB *Nitrosomonas marina*-like AOB	4	2	2	0	13	0	0
Nitrosospira tenuis-like AOB	16	0	0	7	0	62	0
Nitrosomonas europaea-like AOB	0	0	0	1	0	19	0
Nitrosococcus mobilis-like AOB	1	0	0	0	0	0	0
Nitrospira-like NOB	0	2	14	24	51	0	2

[a]Screened by REA

FISH

After sampling, the biomass was immediately processed and fixed in 4% para formaldehyde for 3 h at 4°C. Then the biomass was washed with phosphate buffered saline (pH 7.4) and stored at a 1:1 ratio of phosphate-buffered saline and 100% ethanol at 20°C. In situ hybridizations were performed as described by Manz et al. [31,34]. Fixed cell biomass was spotted onto clean microscope slides, dehydrated (3 min in 50, 80, and 98% ethanol), and air dried. After the addition of the selected probe (5 ng/L^{-1}), slides were incubated at 46°C for 120 min. The probe was added to a hybridization buffer containing 20 mM Tris-HCl (pH 7.4), 0.9 M NaCl, 0.01% SDS, and the appropriate formamide concentration. A stringent wash step followed, using a wash buffer at 48°C for 15 min. The wash solution contained 20 mM Tris-HCl (pH 7.4), 0.01% SDS, and the appropriate NaCl concentration. After washing, the slides were removed and rinsed with distilled water and air dried. The slides were mounted with Citifluor to avoid bleaching and examined with a Axioskop epifluorescent microscope. The presence of Nitrite-Oxidizing Bacteria (NOB) of the genus *Nitrospira* in the flocs was accessed by FISH using a probe previously designed for slot blot analysis [28]. A semi quantitative method was established by visualizing the flocs and expressing the proportion of cells that hybridized to a particular probe in relation to another, more general oligonucleotide probe. Generally, the percentage of cells which hybridized to the AOB strain-specific probes was expressed as the proportion of cells that hybridized to the NITROSO4E or EUB probe. Biomasses were dually stained for AOB-AOB or AOB-NOB analysis and photographed with a Spot SP100 cooled digital color charge-coupled-device camera. Captured images were overlaid in Adobe Photoshop 6.0.

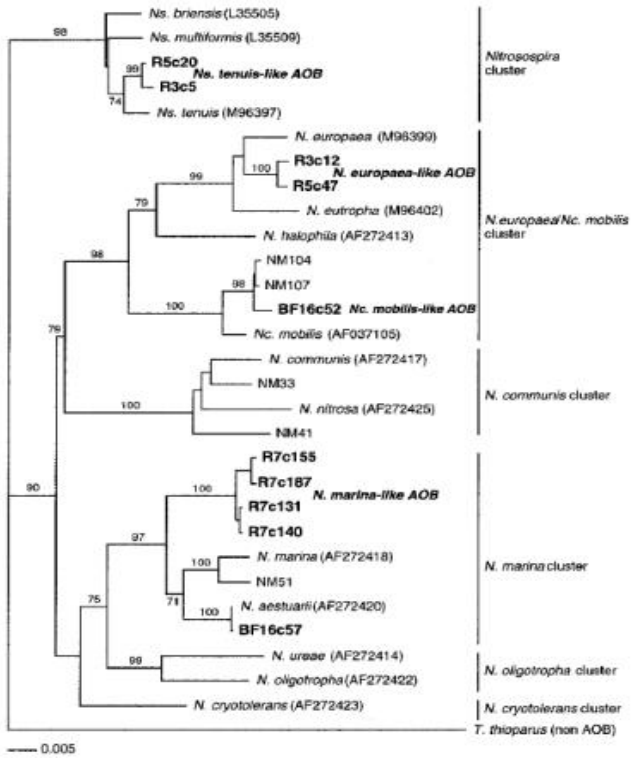

Figure 1: Phylogenetic relationships of the four strains of AOB (in bold) found in this study inferred from comparative analysis of 16S rDNA sequences.

DGGE analysis and profiling

DGGE was performed using a CBS Scientific system, essentially following the method of Ferrari and Hollibaugh [35]. For each sample, 300 ng of PCR product was loaded on a 6.5% polyacrylamide gel with a 52-to- 60% gradient. Gels were run for 15 h at a constant voltage of 75 V in 13 TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA [pH adjusted to 7.4 with acetic acid]) at a constant temperature of 60°C. Gels were scanned using an FMBIO II gel scanner set to measure fluorescein fluorescence. Bands of interest were excised from the gel, and the DNA was eluted from them into 100 ml of water by incubation at 60°C for 2 h. The eluted DNA was purified using Wizard PCR preps (Promega) and sequenced on an automated sequencer (MGIF) with either AM1 or AM2 or both primers.

Results

General clone library analysis

A total of 643 clones were screened from the seven libraries, with 92 clones fully sequenced and another 348 clones partially sequenced (Table 3). Sequencing revealed that the general clone libraries contained bacteria belonging to a number of bacterial phyla, including the *Proteobacteria*, *Cytophagales*, *Actinomycetales*, low-G_C gram-positive bacteria, *Acidobacteria*, *Nitrospira*, OP11, green nonsulfur bacteria, and *Planctomycetales*. The most common clones in each library were affiliated with either *Nitrospira* or *Proteobacteria*. In the libraries BioFarm16, R7, and R5, a large number of proteobacterial clones were shown to be related to known AOB belonging to the _ subdivision of the *Proteobacteria* by BLAST analysis. No clones in any library were related to AOB belonging to the β subdivision of the *Proteobacteria*. Full-length sequencing and subsequent phylogenetic and BLAST analyses of the putative AOB clones showed that there were four strains of AOB in the enrichments which could be grouped in three clusters, using the terminology of Purkhold et al. [34], of the _ subdivision of the *Proteobacteria* (Figure 1) (Table 3). The three clusters were the *Nitrosospira* cluster (*Nitrosospira tenuis*-like AOB), the *Nitrosomonas europaea-Nitrosococcus mobilis* cluster (*Nitrosomonas europaea*-like or *Nitrosococcus mobilis*-like AOB), and the *Nitrosomonas marina* cluster (*Nitrosomonas marina*-like AOB) (Figure 1). A single AOB clone (BF16c57) most similar to *Nitrosomonas aestuarii* was found but not studied any further (Figure 1). In many instances, multiple clones from each AOB strain were identified in a number of the clone libraries (Table 3). All putative AOB clones were partially sequenced to confirm their identity, but only a subset were fully sequenced for phylogenetic and oligonucleotide design purposes. For phylogenetic studies, several fully sequenced clones of three of the AOB strains were randomly chosen. For the *Nitrosococcus mobilis* like AOB, the only clone found was used. Tree topologies generated by PAUP and ARB (not shown) analyses were identical (Figure 1). *Nitrosomonas marina*-like AOB clones, represented by clone R7c140, were found in four of the general clone libraries (Figure 1) (Table 3). Similarity analysis showed this clone sequence to be most similar to *Nitrosomonas marina* (98.8%). *Nitrosomonas marina*-like AOB clones represented 7% of all R7 clones, 2% in the two BC5 clone libraries, and 7% of the BioFarm16 library clones (Table 3). A majority of the *Nitrosomonas marina*- like AOB clones found in the R7 clone library were of a second *Nitrosomonas marina*-like AOB clone sequence (R7c155) (Figure 1). This sequence differed by 5 bases, of over 1,450 bases, from the *Nitrosomonas marina*-like AOB sequence and was not found in the other three clone libraries. Similarity analysis showed the two sequences to be 99.6% similar to each other and most likely represent multiple 16S rDNA operons of the *Nitrosomonas marina*-like AOB. *Nitrosospira tenuis*-like AOB clones (98.8% similar to *Nitrosospira tenuis*) were found in three general clone libraries and represented 30, 19, and 59% of all of the clones in the BioFarm16, R3, and R5 clone libraries, respectively (Table 3). Two of these enrichments were high-ammonia-concentration reactors, while the third enrichment (R3) had been initially maintained as a high-ammonia reactor and then switched to a low-ammonia reactor. *Nitrosomonas europaea*-like AOB clones were found in only two general clone libraries, R3 (3% of all clones) and R5 (18% of all clones). This clone sequence was determined to be 98.4% similar to the sequence of *Nitrosomonas europaea*. A single *Nitrosococcus mobilis*-like AOB clone was found in the high ammonia concentration reactor of BioFarm16 and was determined to be most similar to *Nitrococcus mobilis* (97.6%). The two clone libraries created from the same BC5 biomass (BC5 and BC5) [2] to test the reproducibility of the clone library technique had similar general bacterial diversity (Table 3), and each contained only two *Nitrosomonas marina*-like AOB clones.

Specific AOB clone library analysis

The BioFarm16 and R7 specific clone libraries provided a greater resolution in identifying AOB present in the biomass than the general clone libraries. Phylogenetic analysis of clones from both the specific and general Biofarm16 clone libraries using 640 nucleotides indicated that the clones were nearly identical. Both clone libraries

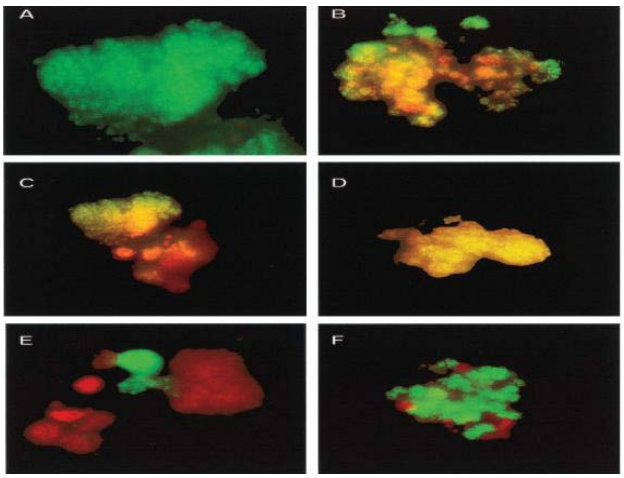

Figure 2: Whole-cell FISH of nitrifying biomasses. (A) FITC stains of *Nitrosomonas marina*-like AOB enrichment to be added to aquaria. (B) Simultaneous hybridization of AOB enrichment with Cy-3 stain for all AOB (red) and FITC stain for *Nitrosomonas marina*-like AOB, resulting in a yellow color for this AOB strain. (C) Simultaneous hybridization of biomass enrichment before addition to a newly established aquarium with Cy-3 stain for all AOB (red) and FITC stain for *Nitrosomonas marina*-like AOB, resulting in a yellow color for *Nitrosomonas marina*-like AOB and showing some non *Nitrosomonas marina*-like AOB. (D) Biomass material harvested from an aquarium with active ammonia oxidation after inoculation with the enrichment from panel C showing the presence of only *Nitrosomonas marina*-like AOB, which are yellow from the simultaneous hybridization with Cy-3 stain for all AOB and FITC stain for *Nitrosomonas marina*-like AOB. (E) Dual staining of nitrifying enrichment with Cy-3 for all AOB and FITC for *Nitrospira* sp. showing the proximity of AOB to NOB. (F) Dual staining of nitrifying biomass with FITC for *Nitrosomonas marina*-like AOB and Cy-3 for *Nitrospira* sp. NOB, elucidating the structure of the nitrifying consortium.

contained *Nitrosomonas marina*-like AOB, *Nitrosospira tenuis*-like AOB, and *Nitrosococcus mobilis*-like AOB. However, REA analysis of the specific BioFarm16 clone library produced five patterns, whereas only three patterns were found in the general BioFarm16 clone library. Sequencing determined that the fourth and fifth patterns were due to the presence of *Nitrosomonas europaea*-like AOB and the second sequence for the *Nitrosomonas marina*-like AOB. Neither of these strains of AOB was detected in the general BioFarm16 clone library. In addition, the AOB diversity of the specific R7 clone library was greater than that of the general R7 clone library. *Nitrosomonas marina*-like AOB were the only AOB identified in the general R7 clone library, whereas the specific R7 clone library identified clones belonging to all four strains of AOB found in this study.

FISH

The NITROSO4E probe, used as a general AOB FISH probe, specifically hybridized to pure cultures of *Nitrosomonas europaea*, *Nitrosospira multiformis*, and *Nitrosomonas cryotolerans* in both single- and dual-hybridization experiments with the AOB-specific Nso190 probe. The NITROSO4E probe yielded an optimal signal at 20% formamide.

Neither the NSMR76 probe, designed for the detection of *Nitrosomonas marina*-like AOB, nor the NSMR34 probe, designed for the detection of *Nitrosospira tenuis*-like AOB, hybridized to pure cultures of *Nitrosomonas europaea*, *Nitrosospira multiformis*, or *Nitrosomonas cryotolerans* at the tested formamide stringencies, demonstrating specificity for their target AOB. The optimal signal for NSMR76 and NSMR34 was determined to be at 20% formamide. The NSMR34 probe did not hybridize to "*Nitrosomonas marina*"-like, *Nitrosomonas europaea*-like, or *Nitrosococcus mobilis*-like AOB cells. The NSMR76 probe did not hybridize to *Nitrosospira tenuis*-like, *Nitrosomonas europaea*-like, or *Nitrosococcus mobilis*-like AOB cells at the tested formamide stringencies (Figure 2).

FISH analysis of biofilms

FISH analysis of the BioFarm16 biomass revealed that about 50% of the EUB-positive cells cohybridized with the general AOB probe. However, only about 2% of these AOB-positive cells were estimated to be *Nitrosomonas marina*-like AOB. The majority (90%) of the AOB-positive cells were *Nitrosospira tenuis*-like, with the remaining 8% or so being unidentifiable with regard to strain type. The BC5 biomass sample was highly autofluorescent. AOB detected with the AOB general probe comprised less than 5% of the EUB-positive cells. Virtually 100% of these cells hybridized with the *Nitrosomonas marina*-like AOB probe. No cells were positive with the specific probes for the other AOB strains found in this study. General AOB probing revealed two distinct AOB strains in the R3 biomass. By using the specific AOB probes in dual hybridization experiments, it was estimated that *Nitrosospira tenuis*-like AOB comprised 90% of the NITROSO4E-positive cells while the remaining 10% of the cells were unidentifiable as to the strain of AOB. No *Nitrosomonas marina*-like AOB positive cells were detected in the R3 biomass. In the R5 reactor sample, over 90% of the EUB-positive cells hybridized to the general AOB FISH probe, indicating a large concentration of AOB cells in this reactor biomass. FISH analysis with the AOB-specific probes demonstrated that 90% of the general AOB-positive cells hybridized with the *Nitrosospira tenuis*-like AOB probe, with the remaining 10% being unidentifiable as to strain type. No *Nitrosomonas marina* like AOB were detected in the R5 biomass by FISH. Only about 10% of the EUB-positive cells in the R7 biomass hybridized to the general AOB probe. More than 95% of these AOB positive cells hybridized with the *Nitrosomonas marina* like AOB-specific probe. *Nitrospira* spp. were found to be in close association with AOB in the biomass from each reactor (Figure 2).

FISH analysis of aquaria inoculated with AOB biomass

The R7PostBA biomass, which was collected from an aquarium inoculated with a biomass dominated by *Nitrosomonas marina*-like AOB, was microbiologically complex, with many bacterial morphotypes hybridizing with the EUB probe. The only AOB strain detected was *Nitrosomonas marina*-like cells. Probing of samples from aquaria inoculated with biomass from reactors BC5 (*Nitrosomonas marina*-like AOB) and R3 (*Nitrosospira tenuis*-like AOB) were also dominated by *Nitrosomonas marina*-like AOB, although the R3 sample did contain some *Nitrosospira tenuis*-like AOB. However, the total percentage of AOB in each sample was 2% or less of the total bacterial community. The biofilm collected from an aquarium inoculated with R5 biomass (*Nitrosospira tenuis*-like AOB) was the only one in which FISH analysis did not detect *Nitrosomonas marina*-like AOB. *Nitrospira* spp. was detected in the biomass from each aquarium by FISH (Figure 2).

Use of AOB-specific PCR primers

The four primer sets developed in this study for specific strains of AOB amplified only their target templates at the optimal annealing

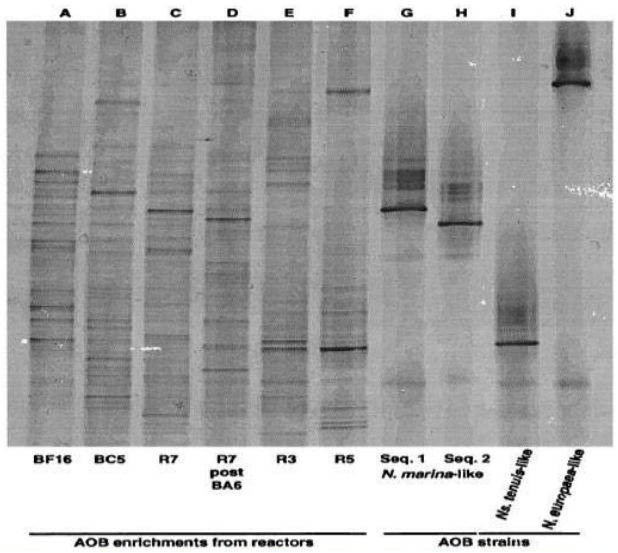

Figure 3: DGGE of AOB enrichments, amplified with a universal and specific eubacterial primer set, from which clone libraries were constructed. Lane D is material collected from an aquarium which was seeded with the R7 enrichment (lane C). Lanes G through J are from clones representing *Nitrosomonas marina*-like AOB (sequence type 1), *Nitrosomonas marina*-like AOB (sequence type 2), *Nitrosospira tenuis*-like AOB, and *Nitrosomonas europaea*-like AOB, respectively.

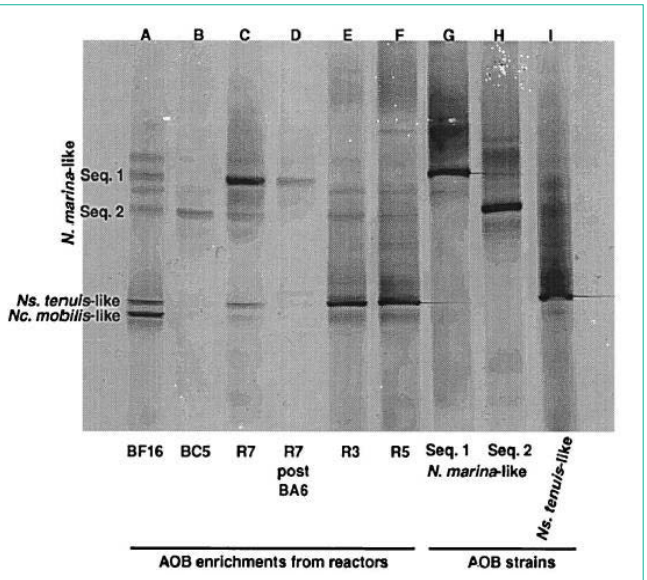

Figure 4: DGGE of AOB enrichments (same samples as in Figure 3) except that the samples were amplified with an AOB-specific primer set. *Nitrosomonas europaea*-like AOB could not be successfully visualized under the gel conditions of this DGGE.

temperature, producing PCR products of the correct size (Table 2). Analysis of the bioreactor biomass with the AOB-specific primer sets showed *Nitrosomonas marina*-like AOB to be present in all bioreactor samples except R5. *Nitrosospira tenuis* like AOB were detected in the BioFarm16, R3, R7, and R5 biomasses but could not be detected in the BC5 sample. No *Nitrosomonas europaea*-like AOB could be detected in biomasses harvested from aquaria inoculated with the various bioreactor enrichments. *Nitrosococcus mobilis*-like AOB were detected only in the BioFarm16 biomass.

DGGE

General DGGE analysis revealed a pattern of multiple bands of various intensities which reflects the complex microbial community in each sample (Figure 3). The sequencing of selected DGGE bands that had migrated the same distance in the gel as the clonal representatives of the AOB strains identified in this study confirmed the presence of the various AOB strains in the bioreactor biomass. The two sequences for *Nitrosomonas marina*-like AOB which differ by only a single base pair in the amplified fragment were easily differentiated in the general DGGE (Figure 3). The AOB-specific DGGE was able to detect three of the four AOB strains found in this study with good spatial resolution (Figure 4). Only the *Nitrosomonas europaea*-like AOB strain could not be reliably detected in the AOB-specific DGGE. Agarose gel analysis of cloned *Nitrosomonas europaea*-like AOB DNA amplified with the specific PCR primers showed a positive reaction (data not shown). However, when the material was run on DGGE, it remained at or near the top of the gel, which may represent a problem with the percentage of denaturant used in the DGGE gel.

Discussion

Amplification of 16S rDNA with specific primers and DGGE have been used successfully in several studies to investigate the diversity of ammonia oxidizers [7,22]. We used a similar approach, employing the nitAB primer set to detect ammonia oxidizers in Arctic Ocean samples and investigating their diversity by DGGE analysis of the nitAB product. The specificity of these primers has been tested [13]. They demonstrated that these primers amplify the 16S rDNA of all nine known species of ammonia oxidizers in the b-*Proteobacteria*. The primers did not amplify the sequences of any closely related, non-ammonia-oxidizing bacteria except *Spirillum volutans*. These primers were used successfully to amplify DNA collected from a permanently ice-covered Antarctic lake where nitrification was evident but from which no ammonia-oxidizing bacteria have been isolated [7,14]. Phylogenetic analysis of the 1.1-kb nucleotide sequences we obtained indicated that they were closely related to ammonia oxidizing b-*Proteobacteria*. None of the sequences we obtained had affinity for non-ammonia-oxidizing bacteria, supporting the specificity of the nitAB primer set. The phylogeny of the four strains of AOB recovered from biomass in this study, with the phylogenetic 16S rRNA tree of Schramm et al. [22] superimposed, is shown in Figure 1. The four strains of this study fall into three of the clusters described by Schramm et al. [22,34]: the *Nitrosomonas marina* cluster, the *Nitrosospira* cluster, and the *Nitrosomonas europaea-Nitrosococcus mobilis* cluster. However, only the *Nitrosospira tenuis*-like AOB, *Nitrosomonas europaea*-like AOB, and *Nitrosococcus mobilis*-like AOB showed a high similarity with previously published AOB sequences (Figure 1). The *Nitrosomonas marina*-like AOB strain from this study likely represents a new species of freshwater AOB, since full length 16S rDNA sequences are only 95% similar to *Nitrosomonas marina*. It has been shown that at 16S rRNA similarity values below 97%, the DNA similarity between two organisms is likely to be less than 70%, and thus, the organisms are probably distinct species [28]. That these criteria apply to AOB belonging to the β subdivision of the class *Proteobacteria* was confirmed by Schramm et al. [22,34]. *Nitrosomonas marina*-like AOB are the bacteria most likely responsible for ammonia oxidation in aquaria, as they were found

by multiple molecular techniques in all but one of the bioreactors maintained at low ammonia concentrations (5 to 10 mg of NH3 N per liter) and enrichments containing *Nitrosomonas marina*-like AOB successfully accelerated nitrification in aquaria. These criteria were not matched by any of the other AOB strains found in this study. In addition, *Nitrosomonas marina*-like AOB were detected by FISH in all the biomasses extracted from nitrifying aquaria accelerated with an enrichment except for one (R5). However, *Nitrosomonas marina*-like AOB seem to represent only a small percentage of the microbial community in an aquaria, as neither they nor any other AOB strain was found in the clone library developed from biomass extracted from a nitrifying aquarium. The relatively low number of *Nitrosomonas marina*-like AOB cells in the microbial community of aquaria may make detection by various molecular methods difficult and could explain why these microorganisms were not previously detected as the putative AOB in freshwater aquaria [12]. PCR amplification under the conditions we used is at best semi quantitative. It is possible (likely) that some samples which did not yield a PCR product with nitAB contain low concentrations of b-proteobacterial ammonia oxidizer DNA. We did not use the nested PCR approach described by Ferrari and Hollibaugh [35] in which nonspecific Bacterial primers are used to increase the relative abundance of Bacterial 16S rDNA in samples prior to a secondary amplification with ammonia oxidizer- specific PCR primers. Figure 2E and F show the close association of AOB and NOB cells with each other in the nitrifying biomass. The association of these two groups of bacteria in the nitrifying flocs points out the difficulty in obtaining pure cultures of AOB. This association has been demonstrated previously in the nutrient-rich environment of wastewater systems [14,31] and is now extended to the comparatively nutrient-poor aquarium systems. Furthermore, the structure of the nitrifying consortium is reminiscent of the consortium of archaea and sulphate reducing bacteria responsible for anaerobic oxidation of methane on the ocean floor [6,25] and would be a good candidate for the further application of coupled FISH and secondary ion mass spectrometry [25]. The topology of the phylogenetic tree, parsimony analysis bootstrap analysis, and similarity matrix analysis suggest that the *Nitrosospira tenuis*-like AOB represent a unique clade which is distinct from *Nitrosospira briensis*, *Nitrosospira multiformis*, and *Nitrosospira tenuis* (Figure 1). *Nitrosospira tenuis*-like AOB grew best in the reactors maintained with a high concentration of ammonia. Nevertheless, enrichments of *Nitrosospira tenuis*-like AOB were able to accelerate nitrification when added to new aquaria. However, *Nitrosospira tenuis* AOB could not be detected by PCR or FISH in the majority of aquarium biomass samples several weeks after being added. Thus, it appears that *Nitrosomonas marina*-like AOB may out complete *Nitrosospira tenuis*-like AOB in the low-ammonia-concentration environment of an aquarium. The *Nitrosomonas europaea*-like AOB are phylogenetically most closely related to *Nitrosomonas europaea* and were found only in reactors with a history of high ammonia concentration. This strain of AOB was also absent in clone libraries and FISH analysis of biomass grown at consistently low ammonia concentrations, suggesting their affinity for high-ammonia-concentration environments. PCR and FISH analyses did, at times, produce conflicting results. In the process of identifying the active AOB in the nitrifying biomass by FISH analysis, it became apparent that some of the results contradicted results of PCR analysis. Two biomass samples which were PCR positive for a specific strain of AOB were negative in the AOB-specific FISH studies (R3 and R7). The occurrence of a positive PCR but negative FISH result can be due to the presence of active but scarce AOB cells, or the PCR result could be a false positive caused by the amplification of DNA from inactive cells or dead cells. The latter can be expected due to the presence of extracellular DNA, which is stable long-term, and the passive dispersal of cells [34]. Under these circumstances, AOB implicated with a positive PCR-negative FISH result in a particular sample could not be absolutely associated with ammonia oxidation. Our results suggest that when there was a conflict, the PCR tests provided a false indication of the presence of an active AOB strain. It was apparent from the results of this study that by altering the ammonia concentrations in the bioreactors, different populations of AOB were generated. The ability to phylogenetically differentiate AOB on the basis of the ambient ammonia concentration has been previously demonstrated under a variety of conditions [10,15,33,35]. Juretschko et al. [11] used maximum ammonia tolerance as one criterion to classify eight new species of AOB. Juretschko et al. [11] examined shifts in the AOB community at ammonium concentrations ranging from 50 to 3,000 mg of N per liter, which overlaps the higher ranges of this study. At these ammonia concentrations, Schramm et al. [22,33] found AOB that fell into the *Nitrosomonas europaea- Nitrococcus mobilis* cluster of Schramm et al. [22,34], which correlates with our results for the *Nitrosomonas europaea*-like and *Nitrosococcus mobilis*-like AOB. Gieseke et al. [10] found a spatial separation of *Nitrosomonas europaea-Nitrosococcus mobilis* cluster AOB and *Nitrosomonas oligotropha* cluster AOB in a phosphate-removing biofilm, with only *Nitrosomonas oligotropha* being present in the deeper (lower-ammonia-concentration) layers of the biofilm, which further supports the possibility of there being physiological differences between the *Nitrosomonas europaea*-like and *Nitrosococcus mobilis*-like AOB found in high-ammonia environments and the low-ammonia *Nitrosomonas marina*-like AOB. That AOB can be phylogenetically differentiated on the basis of the ambient ammonia concentration was also demonstrated by Suwa et al. through isolation studies [35] and 16S rDNA sequence analysis for detecting two general groups of AOB based on their degree of sensitivity to (NH4)2SO4 [35]. The (NH4)2SO4-insensitive strains found by these researchers, which could tolerate (NH4)2SO4 concentrations above 30 mM, would be grouped in the *Nitrosomonas europaea-Nitrosococcus mobilis* cluster of Schramm et al. [22,34], and this compares favourably with our finding that AOB strains from high-ammonia reactors also fall into this cluster. The (NH4)2SO4-sensitive AOB of Ferrari et al. [35], which grew at 3.57 mM (NH4)2SO4 but were inhibited at 10.7 mM (NH4)2SO4, would be grouped in the *Nitrosomonas oligotropha* cluster, which is on the same main branch leading to the *Nitrosomonas marina* cluster containing the *Nitrosomonas marina*-like AOB (low ammonia-concentration AOB) found in this study. When ammonia concentrations were varied, AOB population shifts did occur, thereby altering the presence and activity of important AOB. Low-ammonia environments will likely produce *Nitrosomonas marina*-like AOB, while as the ammonia concentration increases, *Nitrosospira tenuis*-like and *Nitrosomonas europaea*-like AOB will become important until at the highest ammonia concentration *Nitrosococcus mobilis*-like AOB may be predominant. Our results suggest that the AOB found in fish culture environments, such as public aquaria, aquaculture facilities, and home aquaria, where

the ambient ammonia concentration rarely exceeds 5 mg of N per liter, are different from the traditional *Nitrosomonas europaea-Nitrosococcus mobilis* cluster type AOB, which are prevalent in the high-ammonia concentrations typically found in environment such as wastewater and sewage treatment facilities. This, and our results with enrichments of the various strains of AOB in newly set-up aquaria, strongly suggests that start-up inocula for the establishment of nitrification in aquatic culture systems should optimally consist of *Nitrosomonas marina*-like AOB rather than *Nitrosomonas europaea-Nitrosococcus mobilis* cluster AOB.

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