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Using PCR and 16S rDNA Hybridization to Assess for the Presence of *Nitrosococcus oceani* in a Wastewater Treatment Bioreactor

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***Nitrosococcus oceani* of the γ-proteobacteria is known as the only major ammonia-oxidizing bacteria in seawaters. Using species-specific PCR and hybridization, we assayed for this organism in a mixed culture obtained from a wastewater treatment bioreactor, where the source of nitrification was not understood. Presence of *N. oceani* would imply that this population contributed to the high nitrite levels in the bioreactor. Both methods of detection failed to show the presence of the species. We conclude that *N. oceani* was not responsible for the high amount of nitrification. However, we still could not completely rule out the presence of *N. oceani* at some low levels. It was possible that *N. oceani* was undetectable due to the use of non-degenerate primers and unfavorable hybridization conditions.**

The study of ammonia-oxidizing bacteria (AOB) has long been focused on proteobacteria from the β-subdivision. While these bacteria have been detected in both freshwater and marine environments, AOB from the γ-subdivision have only been found in seawater. *Nitrosococcus oceani* and *Nitrosococcus halophilus* are the only known species in the γ-subdivision. More is known about *N. oceani*, which was the first described AOB isolated from seawater (13). The γ-proteobacteria are considered a major AOB group in marine environments. Having been detected in many marine environments at concentrations as high as 10^4 cells ml^{-1} (8, 9, 12, 15), *N. oceani* is the major AOB responsible for the observed nitrification rates in the world oceans. In a wastewater treatment bioreactor, the presence of nitrifying species, including *Nitrospira, Nitrobacter, and Nitrosomonas* were detected by hybridization with probes directed towards the 16s RNA (Rob Simm, personal communication). However, the extent of probe binding did not consistently correlate to the nitrification levels. Even though the bioreactor formed large amounts of nitrate from ammonia and nitrite, binding of the probe to the nitrifying populations was not strong. The inconsistency may be due to mismatches during hybridization, or novel species that had not been detected in the bioreactor before. Since *N. oceani* is a well-known AOB commonly found in seawater, we hypothesized that nitrification in the bioreactor was due to this AOB population from the γ-subdivision.

In this experiment, we assayed for the presence of the halophilic *N. oceani* in the late phase of the bioreactor, where salt concentration was the highest out of all the batch reactors. Diversity of *N. oceani* was recently investigated by Ward and O'Mullan (11) based on the 16S rDNA and *amoA* genes. Great similarity was found between cultivated strains and new strains isolated from a range of marine environments, with more diversity among environmental clones (11). The overall similarity suggests that we would not expect a very divergent *N. oceani* population in our samples. Probes and primers reported in the literature should be sufficient to detect for the species.

MATERIALS AND METHODS

Culture samples and DNA extraction. A mixed population of bacteria was obtained from a sequence batch reactor of wastewater treatment in the late phase. Two replicate samples, each pelleted from 5 ml of bioreactor culture in May 2003, were kindly provided by Rob Simm. The frozen pellet was resuspended in 1.0 ml of extraction buffer (50 mM Tris-BASE at pH 8.0, 5 mM EDTA, 30% sodium dodecylsulfate). DNA was isolated by the method for simultaneous extraction of DNA and RNA (14). DEPC water was not used in the procedure since we were interested in extracting DNA only. To achieve optimal cell breakage, the glass beads (0.1 mm, Sigma) to volume ratio for bead beating by a bead beater (BioSpec) was 50:50. The final pellet was dissolved in 50 µl of TE buffer (10 mM Tris-HCl, 0.1 mM disodium acetate at pH 7.4), and treated with a small volume of 1 mg/ml RibonucleaseA (Sigma) for 15 min at 37ºC. Quality of the isolated DNA was evaluated by spectroscopy (220 nm to 340 nm) and by running the sample in a 1% agarose gel stained with ethidium bromide.

Two-stage PCR amplification. PCR was performed using the AccuPrime™ SuperMix II (Invitrogen) in a Gene Cycler™ Thermal Cycler (BioRad). The first PCR reaction used general primers EUB1 and EUB2, which should amplify any eubacterial 16s ribosomal DNA (3). The maximum amount of the EUB1-EUB2 product was used in the subsequent amplification. The second-stage PCR used a semi-specific set of primers, EUB2 and NOC1, with the latter one being specific for *N. oceani* (6). Reaction conditions (Table 1) were set according to the manufacturer's instructions. DNA products generated were run on a 1% agarose gel to assess amplification.

Table 1. Parameters used in the two-stage PCR amplification.

Primer Sequence (5'→3')	Primers (μM)	Template DNA	Annealing condition	No. of cycles
EUB1: GAGTTTGATCCTGGCTCAG EUB2: AGAAAGGAGGTGATCCAGCC	0.2	200 ng	60°C/ 1 min	30
NOC1: CGTGGGAATCTGGCCTCTAGA EUB2: AGAAAGGAGGTGATCCAGCC	0.2	23 μl	60°C/ 1 min	35

Cloning, hybridization, and sequencing. PCR products were used without further purification for cloning using the TOPO TA Cloning Kit (Invitrogen), with the pCR®2.1-TOPO® plasmid vector and One Shot® Chemically Competent TOP10 *Escherichia coli*. White transformant colonies were patched onto fresh ampicillin selective plates, and transferred onto BioRad nitrocellulose membranes, which were air dried for 1 hour and baked at 80°C for 2 hours under vaccum (1). Each plate was patched onto two nitrocellulose membranes to produce replicates that will be hybridized with different probes. One set of membranes was hybridized with the Nscoc128 probe specific for *N. oceani* and *N. halophilus* (2). The oligo sequence: 5'-CCCCTCTAGAGGCCAGAT-3' was described online by ProbeBase (4). The probe was radioactively labeled with γ-[^{32}P]-ATP using the KinaseMax™ 5'-End-Labeling-Kit (Ambion), and purified with the QIAquick Nucleotide Removal Kit (Qiagen). *Nitrosomonas* in the bioreactor had previously been detected by 16s RNA hybridization (Rob Simm, personal communication). Therefore, the *Nitrosomonas*-specific Nso190 probe was used as a positive control in our 16s rDNA hybridization. Since literature sources on the use of Nscoc128 could not be found, we used previously described formulas to determine the hybridization temperature to be 45°C based on the probe length and composition (1). Nitrocellulose membranes in each hybridization tube were separated by a mesh divider, and incubated in 14 ml of prehybridization solution for 3 hours (1). For hybridization, approximately 30 million CPM of each labeled probe was added directly to the prehybridization solution already in the tube. Each hybridization tube contained one membrane replicate, incubated with either Nscoc128 or Nso190 overnight at 45°C. The washing temperature was also 45°C, in 40 ml of pre-warmed 1X SSC buffer for 10 min, followed by two more 30 min washes. The hybridized membranes were air dried on Whatman 3MM filter paper, and exposed to Kodak X-ray films until appropriate signal intensity developed. Colonies giving positive radioactive signals were streaked onto fresh selective plates. Plasmids from each isolated colony were extracted using the High Purity Plasmid Miniprep System (Gibco Concert). The forward and reverse primers for the cloning plasmid were –21m13 and M13R. Sequencing of the plasmids was done at the UBC Nucleic Acid Protein Services Unit (NAPS). Purified DNA was dissolved in 30 μl of ddH_2O instead of TE buffer, according to NAPS instructions.

Sequence analysis. For each clone, sequences derived from the forward and reverse primers were merged using tools from Pasteur Biological Software (http://bioweb.pasteur.fr/intro-uk.html). Complementary pairing between sequences and probe was analyzed using the matcher program from the same source. NCBI BLAST tools (http://www.ncbi.nlm.nih.gov/BLAST) were used to perform BLASTN search of all sequences. The pairwise alignment of complementary sequences was done also done with the NCBI BLAST (bl2seq). Multiple alignment of the sequences and phylogenetic tree were created with Clustal W (http://www.ebi.ac.uk/clustalw).

RESULTS

Semi-specific PCR reactions using NOC1 and EUB2 primers did not amplify any of the EUB1-EUB2 fragments. However, this was not sufficient evidence to conclude the absence of *Nitrosococcus*, since diversity in the 16S rDNA in the species might cause non-binding with NOC1. Fragments from the first general PCR amplification were cloned into the pCR®2.1-TOPO® plasmid. A total of 200 transformant colonies were transferred to nitrocellulose membranes. Each replicate membrane was individually probed with the Nso190 (control) and Nscoc128. Autoradiographic analysis indicated strong, distinct signals with the Nscoc128 hybridization. Membranes probed with the Nso190 showed much weaker signals and fewer positive colonies. None of the colonies hybridized with both of the probes. Of the 9 positive colonies (out of 200) from the Nscoc128 hybridization, we picked 5 colonies of various intensities for sequencing.

New sequences from the two primers were assembled into one complete sequence. Despite the strong signal with Nscoc128, BLASTN search of sequences from these positive colonies did not show *Nitrosococcus*, but showed a large range of different β-proteobacteria, including *Nitrosomonas*. All matches in the BLAST search had an e-value of 0.0, indicating that identities of the sequences were very possible and that these organisms exist in the bioreactor. Since BLASTN of the five sequences gave various different organisms, it follows that pairwise alignment of the sequences did not show great similarity. The five sequences can be divided into two groups based on their alignment, but their percent similarities were still too low to conclude that any of them were of the same species. A phylogenetic tree (Fig. 1) was constructed based on the organisms with significant matches to the new sequences, along with other previously identified AOB (*Nitrospira* and *Nitrobacter*).

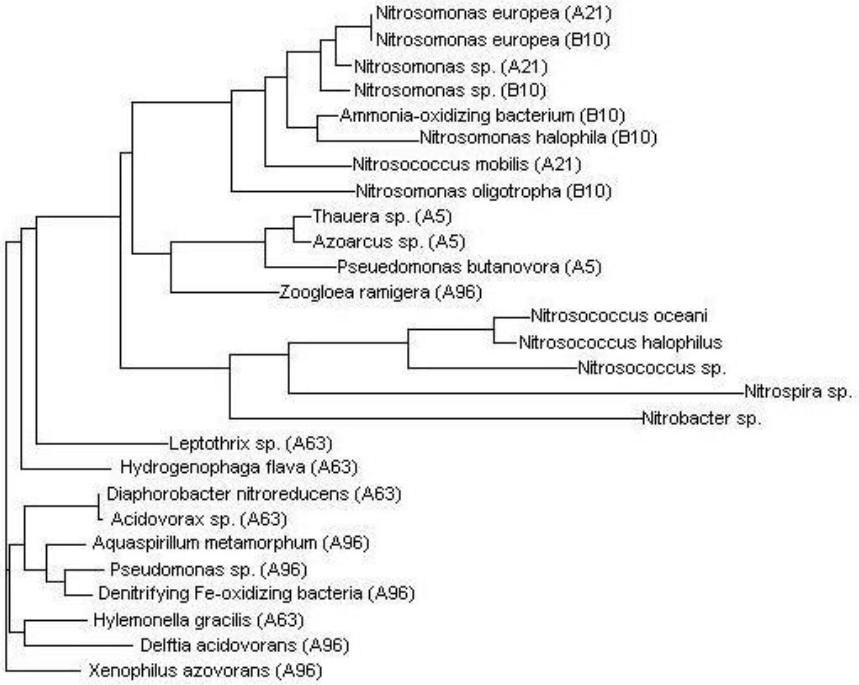

Figure 1. Phylogenetic tree of the 16S rDNA from organisms giving significant matches with the new sequences. Organisms were taken from the BLASTN results of the five new sequences (indicated by the alpha-numeric in parentheses) from positive colonies. The tree was constructed based on neighbor-joining, and reflects the genetic distances between organisms that Nscoc128 had probed for. Other nitrifying bacteria not detected in this experiment, including *Nitrosococcus oceani*, *Nitrospira*, and *Nitrobacter* were also included in the tree for comparison. Their sequences were obtained from NCBI database.

Signal intensity of each colony from which the sequences were obtained was not uniform on the film, even though colonies on the patch plates were of similar size. The differences in signal strength could be due to partial binding or differences in permeability of the cells. A mixed population of cells was also possible since transformant colonies were not streaked and isolated before patching onto membranes for hybridization. Signal strength did not appear to be in correlation with the sequence similarities within the phylogenic tree, or in any relation with *Nitrosococcus*. For example, the signal intensity for colony A21 was a lot stronger than B10, even though their sequences were both identified as *Nitrosomonas europea* in the BLAST search. These two colonies were from different membranes, so the reason for the difference in signal strength could be the membrane being exposed unevenly to the probe during hybridization. Nonetheless, colonies A21 and A63 were on the same membrane and were of similar signal intensity, but their sequences were identified as proteobacteria species that were very distantly related based on the phylogenic tree.

Using the matcher tool from Pasteur Biological Software, local pairwise alignment between the probe and new sequences indicated that there was only a partial binding. All five sequences aligned with the probe sequence in exactly the same manner. Out of the 18 base pairs in the probe, 12 were consecutively complementary to the sequences, with one internal G-T mismatch. Probe specificity was tested by doing a BLASTN search of the oligo sequence, which only gave *Nitrosococcus oceani* strains an e-value of 0.12. The probe was also aligned with *Nitrosomonas* sequences from database, and showed two G-T mismatches, indicating that the affinity was significantly lower.

DISCUSSION

Using PCR as a detection method, *N. oceani* was not detected in the mixed culture. In previous experiments, *N. oceani* had been detected in ice-covered, saline lakes using the NOC1 and NOC2 primers (7). However, the same

primers were not able to detect any *N. oceani* in another saline lake, Mono Lake of California (13). The NOC primers, which should amplify any *Nitrosococcus* DNA, were developed based on only two sequences (6). Therefore, it is possible that other strains of *N. oceani* were not recognized by these primers. If the species is rare and only makes up a small proportion of the AOB in the SBR, then it is also possible that it was not selected for amplification in the initial PCR with the general EUB primers. Consequently, the low levels of *N. oceani* would require some random chance for DNA insertion into the cloning plasmid. Therefore, absence of amplified DNA from the PCR could not rule out the presence of *N. oceani.*

In the hybridization using the *Nitrosococcus*-specific probe, *N. oceani* again was not detected. The false positives on the autoradiograph were due to partial binding of the probe to other bacterial 16s rDNA sequences. Characterization of the probe was not exactly known. Even though specificity of the probe was confirmed by BLAST analysis, other species can still bind imperfectly, especially when specificity of the probe can be assured only when the right conditions are used. The optimal annealing temperature for a primer and its complementary sequence can only be determined experimentally. The temperature we selected was a very rough, general estimate, and created conditions of low stringency. The reduced stringency was also an attempt to increase sensitivity, since absence of the NOC1-EUB2 fragment suggested that *N. oceani,* even if present, would be rare. As a result of the low stringency, probe specificity had to be sacrificed, when other species that were not fully complementary can remain bound to Nscoc128. Using Rychlik's equation (5), we estimated the melting temperature of the probe sequence to be 58^{o}C, which is much higher than the one used in the experiment. Since conditions did not discriminate against other partially complementary species, the hybridization was no longer specific for *N. oceani.* The probe would not be able to find *N. oceani* if it was a small population, or the cell membrane permeability blocked access to the plasmid.

Based on BLASTN search of the Nscoc 128 oligo sequence, the probe should have a higher affinity for *Nitrosococcus* than for *Nitrosomonas*, with one more base pair mismatch in the latter species. However, sequences of the hybridized colonies gave *Nitrosomonas* (sequence A21, B10) as its BLASTN search rather than *Nitrosococcus*. This could be due to the true absence of *Nitrosococcus* in the hybridizing cells, or the loss of probe specificity caused by the non-stringent hybridization temperature.

Despite the false positive results and absence of *Nitrosococcus* using the two detection methods, we still cannot rule out the possibility that *N. oceani* is present at some low level in the bioreactor mixed culture. However, the species being such a small population, we can conclude that *N. oceani* was not a major contributor to the high nitrification in the bioreactor.

FUTURE EXPERIMENTS

The non-specific melting temperature used during hybridization had probably decreased the chances of isolating *N. oceani*. A second attempt can be made using more stringent hybridization conditions. The NOC primers probably limited the diversity of species that could be retrieved. More general primers can be selected to allow for a slightly broader range of detection. Jamie Finley had also probed for *Nitrosomonas* with the Nso 190 probe and sequenced the positive clones. It would be interesting to compare the BLASTN results of his sequences to see if they are closely related to our tentative *Nitrosomonas* sequences which were generated from the Nscoc 128 probe.

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