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Attempted Comparative Hybridization of 16S rRNA and 16S rDNA in Analyzing the Abundance of Nitrifying Bacteria.

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Nitrosomonas europaea **is a commonly studied ammonia-oxidizing bacterium, which oxidizes ammonia into nitrite. The process of nitrification is widely studied due to its importance in the nitrogen cycle, and reduction of ammonia released into the environment. Many techniques, including fluorescence in situ hybridization, have been developed to study the diversity and abundance of *N. europaea* and other nitrifying bacteria, however due to the low growth rates of *N. europaea*, analysis is extremely time consuming. Compartitive 16S rRNA assays using oligonucleotide probes is a more current method used for identifying and determining the abundance of a particular strain. Nevertheless, detection of ribosomal RNA on a membrane can vary depending on the concentration of the organism, probe binding specificities, and various detection techniques. The goal of this study was to create and characterize a 16S rDNA construct, which would act as a positive control in further comparative 16S rRNA assays. pWL061, containing the first 245 base pairs of the *N. europaea* 16S rRNA gene, was digested with *Xmn*1 and loaded onto a Zeta-Probe membrane in increments of 5, 3 and 1 µg. RNA from *N. europaea* and *Escherichia coli* were loaded onto the Zeta-Probe membrane as well. Oligonucleotide probes, Nso190 and Nsm156, which detect ammonium oxidizing β-proteobacteria and *Nitrosomonas* species respectively, were used to detect nucleic acids immobilized on the membrane. While only the 1 µg slot of 16S rDNA from pWL061 was detected by both probes, Nso190 was found to have a much stronger band intensity for 1 µg of rDNA than Nsm156. Neither *E. coli* nor *N. europaea* RNA was detected by either probe.**

Nitrification, the process by which ammonium is oxidized to nitrate, is an important biological process that affects nitrogen in terrestrial, aquatic, and marine ecosystems (14). It is carried out by two groups of autotrophic nitrifying bacteria: ammonia-oxidizing bacteria (AOB), which convert ammonia (NH_4^+) into nitrite (NO_2^-), and nitrite-oxidizing bacteria (NOB) which then convert nitrite into nitrate (NO_3^-) (4, 7, 10). Nitrification has been widely studied because of its importance in the nitrogen cycle. Reduction of ammonia released into aquatic environments reduces the risk of local oxygen depletion, helps to prevent eutrophication, and protects aquatic life (10). As a result, the ability of these organisms to reduce pollutants, makes them attractive organisms for controlled bioremediation in nitrifying soils and waters (8).

Nitrosomonas europaea, an obligate lithoautotrophic ammonia-oxidizing bacterium, is the most commonly isolated and studied ammonium oxidizer (12). *N. europaea* is classified into the ß-subdivision of AOB and has been previously isolated in both fresh water and marine environments. While the ß-subdivision of AOB has been studied more intensely, a second phylogenetic group, the γ-subdivision including *Nitrosococcus oceani* and *Nitrosococcus halophilus*, have been identified and are known only to be found in marine environments (14,16).

The diversity and abundance of ammonium-oxidizing bacteria in natural and engineered environments has been studied using enrichment and isolation techniques, DNA-DNA hybridization studies (10), fluorescence in situ hybridization (FISH) (17), and by immunofluorescenet antibody-based techniques (14). However, because the energy yield from ammonia oxidation is low, leading to small biomass yields with low maximum growth rates, analysis of their environmental diversity is extremely time-consuming (14). Previous studies have utilized immunofluorescence to enumerate nitrifying bacteria, but this technique requires cultivation of a target group to raise antibodies against (6). A common method researchers apply is a comparative 16S ribosomal RNA (rRNA) blot, where RNA from an organism is isolated in immobilized on a membrane, followed by probing with an oligonucleotide for a specific 16S rRNA (15). 16S rRNA is considered a strong genetic marker; considerable variability can be found among organisms with almost identical 16s rDNA genes (2). However, previous studies have detected a consistent discrepancy between nitrification activity and the binding of 16S

rRNA probes of known nitrifiers (7, 10, W. Ramey, personal communication).

This study attempts to create and characterize a 16S rDNA control for the comparision of AOB species using hybridization techniques. The control would be used to determine if there are any discrepancies in sample RNA quality and quantity loaded onto a membrane and whether binding accessibility of each probe differs. The 16S rDNA construct was created by amplification and cloning of the *N. europaea* 16S rRNA gene. RNA was extracted from *Escherichia coli* and *N. europaea* and assayed along with the construct by DNA probes specific to the 16S rRNA of Ammonia-Oxidizing β-Proteobacteria (Nso190) and species of *Nitrosomonas* (Nsm156) to determine if the 16S rDNA construct could be used as a control in further comparative 16S rRNA assays. In theory, a titration of *N. europaea* 16S rDNA of known concentration can be placed on each blot with a sample of pure *N. europaea* rRNA. If the intensity ratio between the *N. europaea* 16S rDNA and 16S rRNA is maintained the same when detected with different probes, then any differences in sample band intensities indicate true concentration differences and are not due to experimental error.

MATERIALS AND METHODS

DNA extraction. A one milliliter volume of pure *N. europaea* culture was divided evenly into two sterile 2 ml screw cap tube. One milliliter of extraction buffer (50 mM Tris HCl pH 8.0, 5 mM EDTA, 3% SDS) and 2 g of 0.1 mm glass beads (Biospec Products, Inc., Bartlesville, OK) were added to each tube. Nucleic acids were then released from the cells by mini bead beating (Mini-BeadBeater 3110BX, Biospec Products, Bartlesville, OK) and precipitated using an ammonium acetate step to remove impurities as previously described (18) except that no diethypyrocarbonate (DEPC) was added to either cell lysis steps. The two tubes were pooled once the nucleic acids were released, prior to the ammonium acetate step. Purified DNA was stored at -20˚C until further use.

PCR amplification and cloning. The extracted *N. europaea* DNA was amplified using generic bacteria primers (synthesized by Nucleic Acids Protein Services Unit (NAPS) of the Biotechnology Laboratory at the University of British Columbia (UBC)), 27f and 1542r with sequences 5'-GAGTTTGATCCTGGCTCAG-3' and 5'-AGAAAGGAGGTGATCCTAGCC-3' corresponding to positions 9-to-27 and 1525-to-1542 of the *E. coli* 16S rDNA, respectively (3,15) (Table 1).

Amplification of the target was performed by PCR following the provided protocol (TOPO TA Cloning Users Manual, Invitrogen, Burlington, ON) using 2.5 µL of each primer (concentration unknown) and approximately 50 ng of template DNA (Invitrogen, Burlington, ON). The amplification cycle was adapted from previous work (3) with the exception of the annealing temperature, which varied from 55.9-64.8°C in 1.8°C increments using the Biometra T Gradient Apparatus (Whatman, Florham Park, NJ). The amplified 16S rDNA from the annealing temperature 63.0°C was directly inserted into pCR®2.1-TOPO® cloning vectors and transformed into chemically competent TOP10 *E. coli* supplied by the TOPO TA cloning kit (Invitrogen, Burlington, ON, Cat# K4500-01) by following provided protocols by the manufacturer (TOPO TA Cloning Users Manual, Invitrogen, Burlington ON). The transformants were selected on Luria-Bertani (LB) (1.0% Tryptone, 0.5% Yeast extract, 1.0% NaCl, pH 7.0, 15 g/L agar) plates containing 50 µg/mL Ampicillin and 40µL of 40mg/mL of X-Gal (Gold Bio Technology Inc., St. Louis, MO) spread on the surface.

Plasmid and RNA isolation. The Fermentas GeneJet Plasmid Miniprep Kit (Burlington, ON, Cat# K0501) was used to isolate plasmid from a 5 mL overnight culture of transformed TOP10 *E. coli*, grown in selective media (LB broth + 100 µg/mL Ampicillin). Sequencing results indicated cloning of *N. europaea* 16S rDNA into pCR®2.1-TOPO® did not work, thus for slot blotting and hybridization plasmid pWL061 was used (FIG.1)

Ribosomal RNA was extracted from a frozen stock of *N. europaea* cells (unknown concentration, vial 1 frozen down by Rob Simm on Dec 12th 2002, vial 2 frozen down by Rob Simm on March 18th 2003). Three hundred micro-liters from each stock vial were added to a sterile 2.0 mL screw top tube along with 320 µL of Tris-buffered phenol (saturated solution, pH <7) and 50 µl 1% sodium dodecyl sulfate (SDS). One- tenth of a millimeter glass beads (BioSpec Products, Inc., Bartlesville, OK) were added until the vial was full. Cells were mechanically disrupted using a Mini-BeadBeater (BioSpec Products, Inc. Bartlesville, OK) at 4800rpm for 3 minutes. Once disrupted, the tubes were heated at 65°C for 5 minutes, vortexing every minute for 20 seconds, followed by a 1 minute incubation on ice. The lysate was then microfuged at 14000 rpm at 4°C for 15 minutes. After microcentrifugation two distinctive layers formed, a bottom organic layer containing the beads and a top aqueous layer. The top aqueous layer, which contained the RNA, was removed into a sterile RNase-free 1.5 mL eppendorf tube and a second extraction was performed by microfuging this sample with an equal volume of phenol: chloroform (pH <7). The top aqueous layer was recovered again and placed in a new sterile RNase-free 1.5 mL eppendorf tube. A third extraction was performed using an equal volume of chloroform, followed by a third microfuge step. The top aqueous layer was placed into a new RNase-free 1.5 mL eppendorf tube again and for every 100 µL of sample, 167 µL of 4 M LiCl stock solution was added to give a final concentration of 2.5 M. The treated samples were stored overnight at -20°C to allow the RNA to precipitate, followed by microfugation and removal of the supernatant. The pellet was washed with 500 µL of 70% Ethanol and allowed to dry at room temperature for approximately 10 minutes. The isolated RNA pellet was stored in DEPC treated water and stored at -80°C until needed. *E. coli* rRNA was extracted along side *N. europaea* RNA using the same methods as described above, except that the starting material, a 4.0 mL overnight *E. coli* culture (strain B23), was microfuged (14000 rpm for 15 minutes, 4˚C) directly in the 2.0 mL screw top tube, the pellet of which was resuspended in 800 µL of DEPC-treated water.

The quality of both plasmid and RNA extractions were determined by taking an absorbance reading of the samples from 200-350 nm. Gel electrophoresis was also performed using 1X TAE buffer (40 mM Tris pH 8.0, 1.14 ml glacial acetic acid, 1 mM EDTA) in a 1.0% agarose gel. The gels were stained using ethidium bromide (0.2µg/mL) and visualized and photographed under a UV transillumiator.

Slot Blotting. RNA and DNA samples were immobilized onto a Zeta-Probe Biofilm membrane (BioRad, Hercules, CA) as per Alkaline RNA and DNA Denaturation and Fixation Protocol in the Bio-Dot SF Microfiltration Apparatus Instruction manual. The DNA constructs were first digested with *Xmn*1 (New England Biolabs, Ipswich, MA) to linearize the plasmid, and then denatured in a final concentration of 0.4 M NaOH, 10 mM EDTA by heating at 100°C for 10 minutes. An equal volume of 2 M ammonium acetate pH 7.0 was added to the denatured DNA sample to neutralize it. The DNA samples were added to the membrane as in Table 2. RNA samples were denatured in a final concentration of ice cold 10 mM NaOH, 1 mM EDTA and immediately applied onto a pre-wet membrane. Vacuum was applied to pull liquid through the membrane. Wells that contained DNA were washed once with 0.4 M NaOH and wells that contained an RNA sample were washed multiple times with 10 mM NaOH, 1 mM EDTA. After blotting the membrane was air-dried and cut in half through lane 3, creating membrane 1 and membrane 2 (see table 2).

Table 1. Names, target positions, sequences and specificities of the probes used in this study.

Probe	*E. coli* 16S rRNA position	Sequences	Probe dissociation temperature (°C)	Targeted groups
Nso 190	190-208	5' - CGATCCCCTGCTTTTCTCC - 3'	55	Ammonia-oxidizing ß-*Proteobacteria* (7)
Nsm 156	156-174	5' - TATTAGCACATCTTTCGAT - 3'	46	*Nitrosomonas C-56, Nitrosomonas europaea, Nitrosomonas eutropha*, and *Nitrosococcus mobilis* (7).

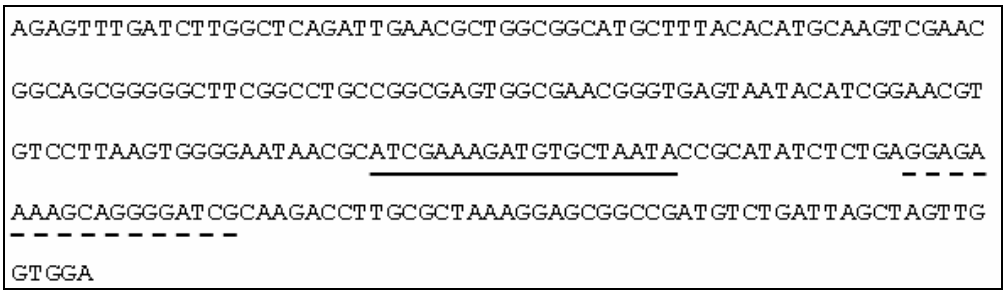

FIG. 1. Sequence of *N. europaea* 16S rRNA from plasmid pWL061 inserted in a pCR®2.1-TOPO® plasmid. The binding site for Nsm156 (solid line) and Nso190 (dashed line) have been noted.

Table 2. Schematic layout of the slot blot membrane. The amount and identity of each sample is clearly labeled. The membrane was cut into two with part (a) becoming membrane 1 and part (b) becoming membrane 2.

(a) | (b)

	1	2	3	4	5
A	blank	*E.coli* RNA 5µg	blank	*E.coli* RNA 5µg	blank
B	*N.europaea* DNA 5µg	*E.coli* RNA 4µg	blank	*E.coli* RNA 4µg	*N.europaea* DNA 5µg
C	*N.europaea* DNA 3µg	*E.coli* RNA 3µg	blank	*E.coli* RNA 3µg	*N.europaea* DNA 3µg
D	*N.europaea* DNA 1µg	*E.coli* RNA 2µg	*N.europaea* RNA 0.5µg	*E.coli* RNA 2µg	*N.europaea* DNA 1µg
E	blank	*E.coli* RNA 1µg	blank	*E.coli* RNA 1µg	blank

Hybridization. Hybridization Protocols for DNA or RNA Bound to Nitrocellulose or Zeta-Probe Membrane was followed in the Bio-Dot SF Microfiltration Apparatus Instruction manual. Due to the limited *N. europae*a RNA, the membrane was cut in half through lane three. Each half of the membrane underwent the same hybridization process with the exception that membrane 1 was probed with Nso190 and membrane 2 was probed with Nsm156 (see Table 1 for sequence identities). The air-dried membranes were placed in prewarmed prehybridization (1 mM EDTA, 0.5 M Na_2HPO_4 pH 7.2, 7% SDS) solution and left rotating overnight in a 40˚C oven. The prehybridization solution was changed and denatured probes were then added at 800 ng/mL and incubated at 40˚C overnight. After hybridization, membranes were washed twice in washing solution A (1 mM EDTA, 40 mM Na_2HPO_4 pH 7.2, 5% SDS) for 45 minutes per wash at room temperature. Followed by two washes in washing solution B (1 mM EDTA, 40 mM Na_2HPO_4 pH 7.2, 1% SDS) for 15 minutes per wash at 55°C for Nso190 probe and 46°C for Nsm156 probe. The membranes were rinsed briefly with DEPC-treated maleic acid solution (0.1 M maleic acid, 0.15 M NaCl pH 7.5) and then blocked for 30 minutes at room temperature in 10 mL of 1% Western Blocking Solution (Roche, Cat# 1921673). Membranes were then incubated in Stepavidin-POD (Roche, Cat# 1108915301) diluted in 1% Western Blocking Solution at 10 U/mL for 30 minutes at room temperature according to the manufacturer directions (Roche, Laval, QB.). The membranes were washed three

times at room temperature with DEPC-treated maleic acid solution containing 0.3% Tween for 15 minutes followed by rinsing in maleic acid solution alone.

Chemilluminesence Detection. The membranes were rinsed briefly in DEPC- treated water and placed on SaranWrap® in a dark room. A total of 5.0 mL of Lumi-Light Enhancer was mixed with 5.0 mL of Lumi-Light Stable Peroxide Solution (Roche Applied Science, Lumi-Light Western Blotting Substrate, Cat #12015200001) and then under red light, immediately added equally to each membrane. The detection solution was incubated for 5 minutes in the dark then taken out of the detection solution and placed on a new SaranWrap®. The membranes were then exposed to audioradiography film (Bioflex MRI Film, Clonex Corporation) for intervals of 1, 2.5, 5 and 10 minutes.

RESULTS

RNA isolation and quantification. A total of 98.3 µg of *E. coli* RNA was isolated from 4 ml of an overnight culture of *E. coli*. After agarose gel electrophoresis, the isolation showed two bands corresponding to 23S and 16S rRNA (FIG.2). The RNA preparation from a frozen sample of *N. europaea* yielded 0.435 µg of rRNA and two bands corresponding to 23S and 16S were identified after electrophoresis (FIG.3). The overall quality of both *E. coli* and *N. europaea* rRNA was determined using spectrophometric methods. The ratio of A_{260}/A_{280} of the *E. coli* rRNA was 1.63, which is marginally acceptable, indicating high protein levels in the isolated rRNA sample; however the bands show the degree of degradation was small. The A_{260}/A_{280} ratio of *N. europaea* rRNA was unacceptable at 1.34. The 23S band appears to be degradated (FIG.3), indicating the presence of RNases or other denaturing components in the extracted sample. The concentration of *N. europaea* RNA was determined as a relative estimate of the band intensity following the gel electrophoresis since the spectrophotometric ratio was so low.

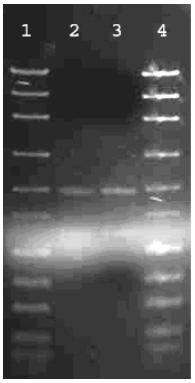

FIG. 2. Agarose gel electrophoresis of isolated *E. coli* rRNA to confirm the quality of the rRNA. Lane 1 and 4 contain MassRuler™ Express DNA Ladder Mix, Forward, ready-to-use (Fermentas), at 5 µl and 10 µl loaded respectively. Lanes 2 and 3 correspond to the isolated rRNA from *E. coli* with ~1 µg loaded in each lane. The gel was run at 105V for 56 minutes, then stained with ethidium bromide (0.2µg/mL) for 10 minutes and destained in DEPC-treated water prior to capturing the image.

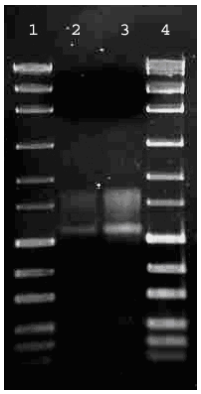

FIG. 3. Agarose gel electrophoresis of isolated *N. europaea* RNA to confirm the quality of the rRNA. A total of 5 µl and 10µl of MassRuler™ Express DNA Ladder Mix, Forward, ready-to-use (Fermentas), standard were loaded in lane 1 and 4, respectively. Lanes 3 and 4 correspond to 1/100 and 1/20 of the total isolated volume of rRNA, correspondingly. The gel was run at 90V for 70 minutes, then stained with ethidium bromide (0.2µg/mL) for 10 minutes prior to capturing the image.

Preparation of Plasmid DNA for Slot Blotting. A total of 20.5 µg of plasmid pWL061 was isolated using the GeneJet plasmid Miniprep Kit (Fermentas) from a 5 ml overnight culture of transformed DH5 α-T1 *E. coli*. Plasmid quality was an acceptable A_{260}/A_{280} ratio of 1.90. The isolated plasmid was then digested with the 100 units of *Xmn*1 in a total volume of 400 µl for 1 hour and 43 minutes to linearize the plasmid. As shown in Figure 4, the digestion reaction went nearly to completion. The *Xmn*1 digested form of the plasmid was denatured and loaded onto the membrane via slot blotting.

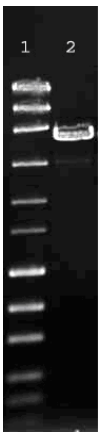

FIG. 4. Agarose gel electrophoresis of *Xmn*1 digested pWL061 to confirm complete digestion of the plasmid. Lane 1 contains 10 µl of ™ Express DNA Ladder Mix, Forward, ready-to-use, standard (Fermentas) and lane 2 contains ~0.42 µg of digested plasmid. The size of pWL061 is ~ 4.2 kB. The gel was run at 90V for 70 minutes, then stained with ethidium bromide (0.2µg/mL) for 10 minutes prior to capturing the image.

Hybridization autoradiogram. As indicated in Table 1, Nso190 should bind to 16S rRNA from all ammonia oxidizing ß-proteobacteria, while Nsm156 is specific to 4 species of ammonia oxidizing bacteria, including *N. europaea*. Membrane 1 was incubated with Nso190 and membrane 2 was incubated with Nsm156 and then both membranes were exposed to chemiluminescent detection solution. Only one band on each membrane corresponding to 1 µg of rDNA was detected (FIG. 5 & 6) (see Table 2 for band identity). No bands were detected in the negative control wells, the wells containing a higher concentration of rDNA (3µg and 5µg), or in the *N. europaea* rRNA well (3D).

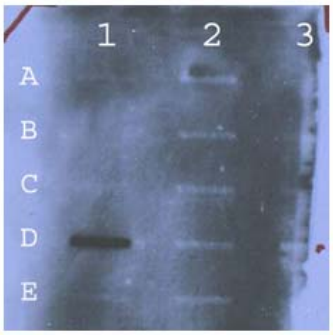

FIG. 5. Autoradiogram of hybridized Nso190 onto membrane 1 (2.5 minute exposure). Refer to table 2 for identity of bands. Well D1, 1 µg rDNA, was the only sample detected; the remaining white bands are simply impressions in the membrane, and not detected samples.

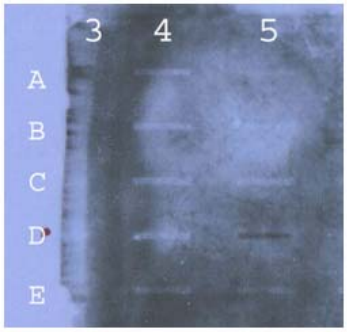

FIG. 6. Autoradiogram of hybridized Nsm156 onto membrane 2 (2.5 minute exposure). Refer to table 2 for identity of bands. Well D5, 1 µg rDNA, was the only sample detected; the remaining white bands are simply impressions in the membrane, and not detected samples.

DISCUSSION

The goal of this study was to create a construct containing the *N. europaea* 16S rRNA gene sequence and use it to detect differences in nitrifying bacteria abundance via comparative 16S rRNA hybridization assays. There were many difficulties that arose when constructing the 16S rDNA construct from scratch. First, *N. europaea*, are extremely slow growing and do not pellet when centrifuged, making them hard to cultivate and grow in large quantities to extract sufficient DNA/RNA for further study. Secondly, since the 16S rRNA gene is highly conserved among microbial species, it is hard to differentiate PCR amplified sequences between *N. europaea* and possible contaminants (2). Therefore, when the quality of the amplified product is analyzed using agarose gel electrophoresis, there could be contaminating 16s rRNA present. As a result of these difficulties, plasmid pWL061 was used as the *N. europaea* 16S rDNA control in slot blotting and hybridization. pWL061 contains the first 245 base pairs of the *N. europaea* 16S rRNA gene within the pCR®2.1-TOPO® vector (FIG. 1). This shorter sub-region of the 16S rRNA gene only contains binding regions for the previously described Nso190 and Nsm156 probes (7).

Prior to slot blotting, pWL061 was digested with *Xmn*1 (FIG.4). As shown by gel electrophoresis, the restriction enzyme digest went nearly to completion, and this forms the basis for assuming that the probes should have had access to their binding sites on the 16S rDNA construct. Digesting pWL061 with the restriction enzyme was necessary to linearize the plasmid to prevent re-coiling during the time between denaturation and the actual blotting procedure. Hybridization of the linearized pWL061 was imperative, since it allows for the probes to access their binding sites. If the plasmid were supercoiled, then the probe binding would be based on access rather then affinity, preventing an even distribution of probe binding (1).

From the autoradiogram, it is evident that the two probes, Nso190 and Nsm156, have very different binding affinities to *N. europaea* rDNA in the blot analysis. This is shown in Figures 5 & 6 where binding of Nso190 to 1µg of rDNA produced a stronger band intensity then Nsm156. Since both membranes had equal amounts of 16S rDNA loaded into each slot, these results suggest that Nso190 is more sensitive to *N. europaea* 16S rDNA. Possible reasons for this outcome could be higher G+C content in Nso 190, leading to a stronger interaction with the immobilized nucleic acid, (13) or non-optimal hybridizing or washing conditions for Nsm156. The binding of probes is dependent on many factors including temperature, NaCl concentration, and formamide concentration of the wash solutions (5). Salt and formamide concentrations should not have played a role in the probe binding, since neither regent was used in the wash buffer. Thus, the higher sensitivity of Nso 190 is most likely due to the lower washing temperature used during hybridization. Previously, Ng discussed the probe dissociation temperature for Nso190 being lower than described in other literature (7,8). Hence, the stronger binding intensity of Nso190

with the lower wash temperature in this study supports Ng's conclusions.

There are discrepancies in the audiogram results. For example, only the 1µg slot of *N. europaea* rDNA was detected by Nso190 and Nsm156 and neither of the higher concentration slots of *N. europaea* rDNA were detected by chemiluminescence (FIG 5 & 6). This indicates that the slot blotting procedure may be been performed incorrectly, or there may have been problems within the membrane itself. Both of the higher rDNA samples took a long time to be pulled onto the membrane. It is possible that during the procedure, the DNA at higher concentrations had time to renature and form a double stranded plasmid. Higher concentrations lead to slow filtrations, allowing for the time to renature. The ability of probes to reach their target sites within a three dimensional structure is a major factor for hybridization efficiency (1). The lower concentration DNA sample of 1 µg, was rapidly incorporated into the membrane and did not have time to reanneal. Since the positive control did not work as expected, it cannot be concluded if the negative control, *E. coli* rRNA, worked as intended.

It is of interest that neither probe was sensitive to the *N. europaea* rRNA. As described by Ng (8), the Nso190 probe is sensitive to *Nitrosomonas* RNA at 5 µg, while Nsm156 is sensitive to amounts of RNA less then 0.5 µg (8). Since the amount of *N. europaea* rRNA loaded onto the membrane was 0.5 µg, one would expect that Nsm156 would bind to the rRNA. The concentration of *N. europaea* rRNA was determined using agarose gel electrophoresis rather then using absorbance readings. This was done because the amount of extracted RNA was very low, yielding low absorbance values within error limits. Therefore, it is possible that the amount loaded onto the membrane was much lower then calculated, leading to a lack of detection by either probe.

This study shows that the rRNA probes Nso190 and Nsm156, which have previously been used to probe and detect RNA, can bind the 16S rDNA construct with different affinities. In theory, the band intensities for Nso190 and Nsm156 should be equal when detecting the construct, but results indicated binding differences. *N. europaea* rRNA was not detected with either probe, which contradicts previous studies, and highlights the sensitive nature of RNA, which makes it difficult to be used in hybridization experiments. Based on the unexpected binding pattern of the rDNA control, it is hypothesized that the lack of RNA detection was due to membrane binding issues.

FUTURE EXPERIMENTS

Since this study did not yield conclusive results to whether the ratio of 16S rDNA and 16S RNA from the same species can be used as a control in comparative 16S rRNA experiments, it would be ideal to repeat this study to get the proper autoradiogram supporting the hypothesis. It would also be necessary to determine why the higher concentrations of rDNA were not detected on the membrane, whether it is due to the type of membrane used or whether the slot blot procedure must be optimized to blot higher concentration of nucleic acid. To test whether RNA was not degraded during the blotting or hybridization steps, the membrane could be stripped and probe with a universal probed such as EUB388 or UNIV1392 to determine if RNA is present (9).

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