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Detection of the Presence of γ Subclass Proteobacteria in a Sequencing Batch Reactor and the Determination of Probe Dissociation Temperatures for Nsc128 and Nso190

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We tested the presence of γ and β subclass of the *Proteobacteria* in samples collected on different dates from a Sequencing Batch Reactor fed with ammonium as the primary energy source. The bacteria were identified by the 16S rRNA-targeted oligonucleotide DNA probes Nsc128 and Nso190 respectively. *Nitrosococcus halophilus* and *N.oceani*, which belong to the γ subclass of the *Proteobacteria*, were absent from the samples as no signal was detected using the Nsc128 probe. Therefore, *Nitrosococcus* of the γ subclass of the *Proteobacteria* did not contribute to the continuous production of nitrite in the bioreactor. Isolated RNA samples that hybridized with Nso190 were used to determine the probe dissociation temperature. The experimental probe dissociation temperature ranged from 44.1°C to 51.9°C, which deviated from the predicted melting temperature (60°C). The lower experimental dissociation temperatures might be due to the incomplete matching of Nso190 and the 16 rRNA sequence of the targeted bacteria.

Nitrification is an important biological process carried out by two groups of autotrophic nitrifying bacteria. The process is divided into two steps. Ammonia is oxidized to nitrite by ammonia-oxidizing bacteria (AOB) while nitrite is converted to nitrate by nitrite-oxidizing bacteria (NOB) (1, 5). This process has been widely studied by scientists because it belongs to part of the nitrogen cycle. Nitrifying bacteria can be beneficial to the environment as they play an important role in sewage treatment plant in which they help in reducing the toxic ammonia content of wastewater (8). On the other hand, they cause pollution by converting ammonia from fertilizers to nitrite and nitrate in water, or by producing nitric oxide that causes greenhouse effect (2, 10).

Nitrification can be investigated by setting up bioreactors that contain nitrifying bacteria with appropriate medium and living conditions. However, recent studies (Rob Simm, personal communication) revealed that the amount of nitrite and nitrate produced by the bacteria in the bioreactor did not correspond to the amount of bacteria present in the system. The levels of nitrite and nitrate stayed high even though the binding of probes directed towards the 16S rRNA of the nitrifying bacteria was low. There are two possible reasons for the inconsistencies: mismatches between the probes and bacterial 16S rRNA sequences or the presence of novel nitrifying bacteria in the bioreactor that cannot be detected by the designed probes.

The aims of this experiment are to detect the potential novel AOB that might be responsible for the first step of nitrification and to determine the probe dissociation temperatures for both Nsc128 and Nso190. Many studies that investigated nitrification involved the examination of β subclass but not the γ subclass of the *Proteobacteria* because the only known nitrifiers in the γ subdivision consists of *Nitrosococcus halophilus* and *N.oceani*, no other ammonia oxidizer within the subclass have been described (7). According to the study (Rob Simm, personal communication), the amount of ammonium converted to nitrite remained high although the population of the detected ammonia oxidizers by Nso190 probe decreased. We postulated that the γ subclass of the *Proteobacteria* might be the novel bacteria present in the Sequencing Batch Reactor (SBR) and might be responsible for the high nitrite level. Nso190 is unable to detect the γ subclass *Proteobacteria* because it is specific for the β subclass *Proteobacteria*. However, few specific probes have been reported in the literature for the γ subclass *Proteobacteria*. The unpublished Nsc128 probe designed by Juretschko (4, 6) was used for the detection of *N.halophilus* and *N.oceani* in the SBR of this experiment.

MATERIALS AND METHODS

Collection and Storage of Sample. The ocean sample collected from the Jericho Beach (October 27, 2003) was used as the control of this experiment as it is believed that *Nitrosococcus oceani* is the major ammonia-oxidizing bacteria (AOB) group in seawater (13). Bacterial cells from the ocean sample were filtered through the 0.22 μm type GS Millipore® filter membrane using vacuum. Plastic spatula and 1.5 ml of filtered ocean water were used to remove the trapped bacterial cells from the filter membrane. Filtered ocean water was used instead of deionized

water for washing the membrane to prevent cell lysis. The washes were collected and stored for RNA isolation. Samples were collected at different dates from the SBR, pelleted and stored at –80°C. The frozen samples used in this experiment were collected on March 15, March 24, March 26, April 21 and May 24, 2003.

RNA isolation. RNA was isolated from the samples by extraction using guanidinium thiocyanate-acidic phenol-chloroform (Trizol Reagent) using the beat-beating method. Two ml of Trizol Reagent was added to each of the thawed samples and then thoroughly mixed. One ml of the mixture was added to a RNase free bead beater vial. Then 0.2 ml of chloroform was added before filling the vial with glass beads (0.1 mm diameter, Sigma). The bead beating was carried out at 4°C for 3 minutes at 4800 rpm. According to the manufacturers instructions (BioSpec), the vial was cooled on ice for 1 minute after each minute of beating. The top aqueous phase containing the RNA was transferred to a new tube and 0.5 ml of isopropanol was added to precipitate the RNA. The mixture was incubated at room temperature for 10 minutes followed by centrifugation at 14,000 rpm for 8 minutes at 4°C. After removing the supernatant, the pellet was washed with 1 ml 75% ethanol and centrifuged at 10,000 rpm for 5 minutes at 4°C. The supernatant was removed and the RNA pellet was briefly air dried at room temperature for 3-5 minutes. The pellet was then dissolved in 0.1 ml DEPC treated water by passing the solution a few times through the pipet tip. The sample was incubated at 60°C for 10-15 minutes and diluted before measuring the absorption spectrum of RNA at 220 nm to 340 nm. The readings obtained at the wavelength of 260 nm and 280 nm were used for calculating the estimated concentration of the sample. The integrity of the isolated RNA was checked by agarose gel electrophoresis followed by staining with ethidium bromide (9).

Oligonucleotide probes. The following oligonucleotide probes were used for targeting 16S rRNA sequences: (i) Nso190, specific for the ammonia oxidizer in the β subclass of proteobacteria (7); (ii) Nsc128, specific for the *Nitrosococcus halophilus* and *N.oceani*, which belong to the gammaproteobacteria (4, 6). Oligonucleotide probes were obtained from the Nucleic Acids Protein Services (NAPS) Unit at the University of British Columbia. Table 1 shows the sequences and characteristics of the two probes.

^{32}P labeling of oligonucleotide probes. The probes were labeled using the standard protocol from Ambion (KinaseMax™ 5'end-labelling kit). Two pmol of an oligonucleotide probe was labeled in a 20 µl reaction mixture containing 1 µl of T4 DNA polynucleotide kinase, 5.0 µl of [γ-^{32}P]ATP, and 10x kinase buffer at 37°C for 1 hour. This kinase reaction was followed by QIAquick nucleotide removal kit procedure (Qiagen).

Slot Blot Hybridization. The isolated RNA from each sample was diluted to a concentration of 1 µg/200 µl then blotted onto a BioRad Zeta-Probe membrane (9 x 12 cm) using a BioRad slot blot hybridization apparatus. The BioRad standard protocol was used for the slot blot hybridization. After applying the RNA to the membrane, the membrane was baked at 80°C for 30 minutes and stored in a plastic bag at room temperature until probe hybridization. The membrane was cut in half for hybridization with the 2 probes. Prehybridization, hybridization, and washes were done in the Lab-line Instrument Hybridization Incubator, Model 308. During hybridization, the membrane (9 x 6 cm) was hybridized overnight at 40°C in 7 ml hybridization buffer with labeled probe. The hybridization buffer consisted of 0.9 M NaCl, 50 mM $NaPO_4$, 5 mM EDTA, 10x Denhardt's solution, 0.5% SDS, and 0.5 mg of poly(A) per ml. The membrane was washed twice with approximately 70 ml hybridization wash buffer (1x SSC-1% SDS) at 40°C in the oven for 30 minutes for each wash. The radioactive membrane was exposed to Kodak™ BioMax Film overnight at room temperature.

Determination of probe dissociation temperature (T_d). After autoradiography, the areas on the membrane corresponding to the positive signals on the film were cut out and placed into 0.65 ml ClickSeal Micro-centrifuge tubes. Hybridization wash buffer (0.5 ml) was added to each tube. The probe dissociation temperature was determined by raising the temperature from 40°C to 80°C in 5°C increments. The range is approximately 20°C above and below the predicted melting temperature (Table 1). The tubes were warmed in a pre-heated PerkinElmer DNA Thermal Cycler at 40°C for 30 minutes. Each strip was transferred to a new tube filled with 0.5 ml pre-heated fresh wash buffer. The new tube was heated to the next temperature and incubated for 30 minutes. The steps were repeated until the last temperature (80°C) was reached. The wash buffer from the previous micro-centrifuge tube was added to the scintillation vial with 1.25 ml scintillation fluid (Ultima Gold, Packard). The radioactivity in the wash buffer was determined by liquid scintillation counting. The probe dissociation temperature was determined by plotting a graph with % eluted CPM of the wash buffer against the temperature range of the experiment. The dissociation temperature is the point at which 50% of the radioactivity is eluted.

Table 1. Sequences, length, and predicted melting temperature of oligonucleotide probes used in this study

Probe	Sequence	Length (bases)	Predicted Melting Temperature* (°C)
Nso190	5'CGATCCCCTGCTTTTCTCC3'	19	60
Nsc128	5'CCCCTCTAGAGGCCAGAT3'	18	58

*The melting temperature (T_m) was calculated using the formula: T_m (°C) = [2 x (A + T)] + [4 x (G + C)] (9)

RESULTS

Agarose gel electrophoresis and absorption spectrum measurement. The integrities of the total RNA isolated from the bacteria in the 6 samples were checked by agarose gel electrophoresis. The results showed that there were more bacterial RNA in the samples collected on March 24, March 26, and May 24 than in the samples collected on March 15 and April 21. No bands were shown for the ocean sample, which indicated that the concentration of the total bacterial RNA isolated from the ocean sample was low. The RNA concentrations of the samples were determined by measuring the absorption spectrum using spectrophotometer. The estimated concentrations of the 6 samples were calculated and shown in Table 2. Although some of the samples had lower RNA concentrations than the others, all samples (except for the ocean sample) contained sufficient amount of total RNA for the slot blot hybridization experiment.

Table 2. The estimated total RNA concentrations of the 6 samples

Sample	Estimated total RNA concentration* (μg/μl)
March 15	0.873
March 24	1.555
March 26	1.533
April 21	0.766
May 24	1.190
Ocean (Control)	0.066

*****The total RNA concentration was calculated using the formula: RNA concentration = $0.063 \times A_{260} - 0.036 \times A_{280}$

Slot blot hybridization. The radioactively labeled probes, Nso190 and Nsc128, were used to quantify the amount of 16S rRNA of *Nitrosomonas* and *Nitrosococcus* respectively in the total RNA isolated from the mixed bacterial culture. In the first exposure some faint bands were observed on the autoradiograph of the membrane hybridized with Nso190 (Fig. 1) but nothing was shown for the membrane with Nsc128. However, not all samples that were supposed to have bands showed positive results. There were only bands for sample in rows 1, 3,4,5, and the first 2 samples in row 8. Figure 2 shows the autoradiographs of a longer exposure (24 days) of the two membranes. No bands were observed for the membrane with Nsc128 probe while most bands were shown for the membrane with Nso190 probe. However, there were still no bands observed for sample 2 in Fig. 2b, and the bands from different samples had different intensities.

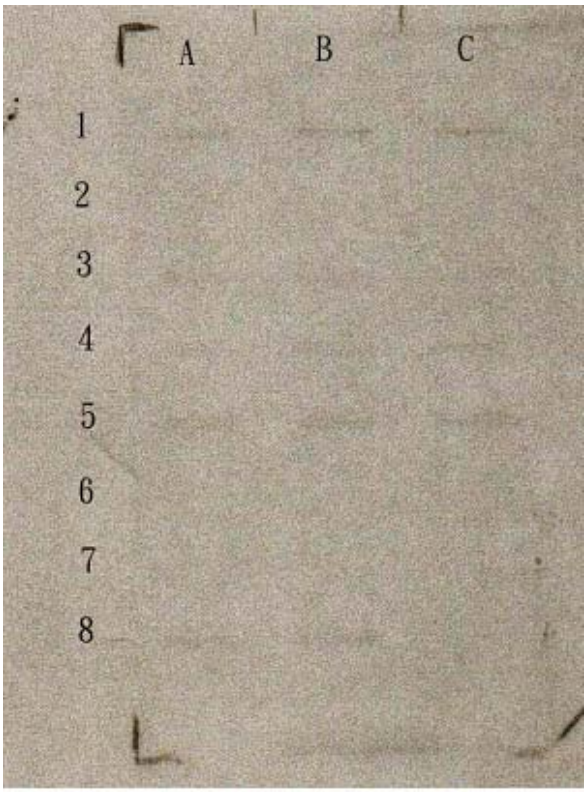
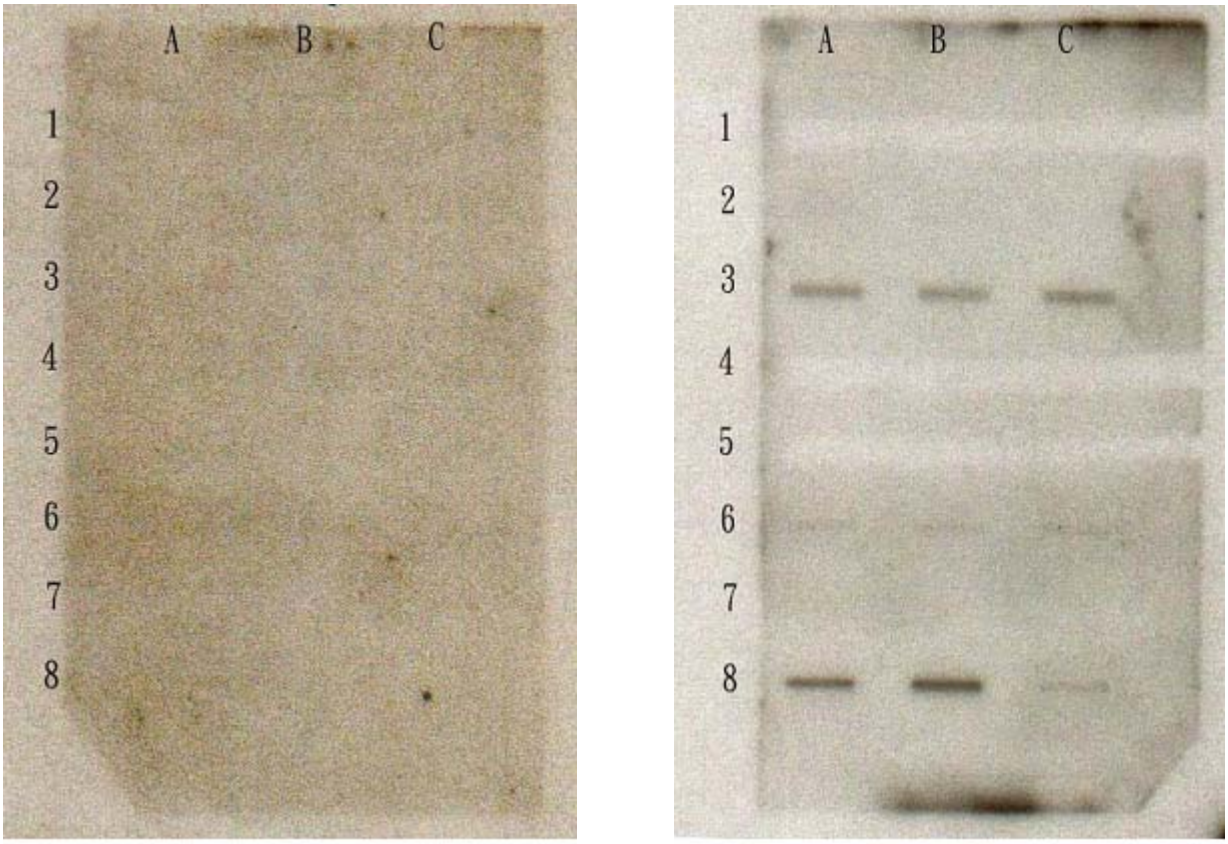


Figure 1. Autoradiograph of Zeta-probe membrane hybridized with radioactive Nso190 probe. Row 1-8 contained total RNA isolated from samples collected on different dates from the SBR. Columns A-C were triplicates for each RNA sample of each row except row 8. The samples were blotted in the following order: 1: March 15; 2: March 24; 3: March 26; 4: April 21; 5: May 24; 6: Control (Ocean sample); 7: Blank (DEPC water); 8: Assorted samples (8A April 21, 8B May 24, 8C Control).



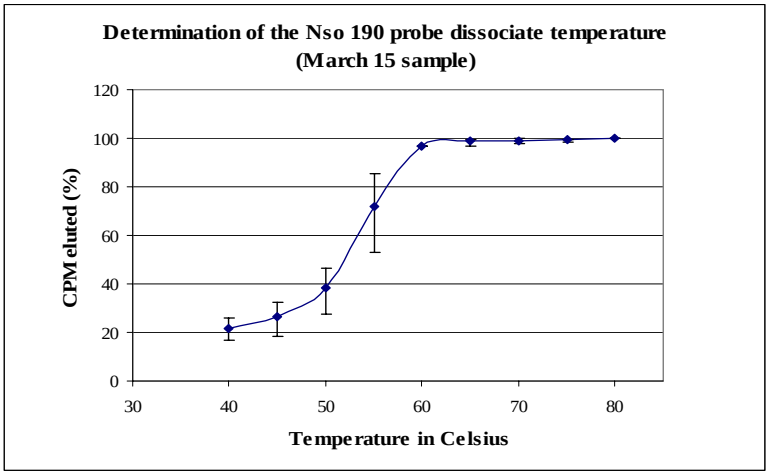
(a) **(b)**

Figure 2. Autoradiographs of Zeta-probe membranes hybridized with Nsc128 (a) and Nso190 (b). Refer to Fig. 1 for annotation of the figures. Membrane areas with sample in rows 1,4,5 were cut out to perform probe dissociation temperature experiment. The remaining membranes were exposed to the X-ray film for 24 days.

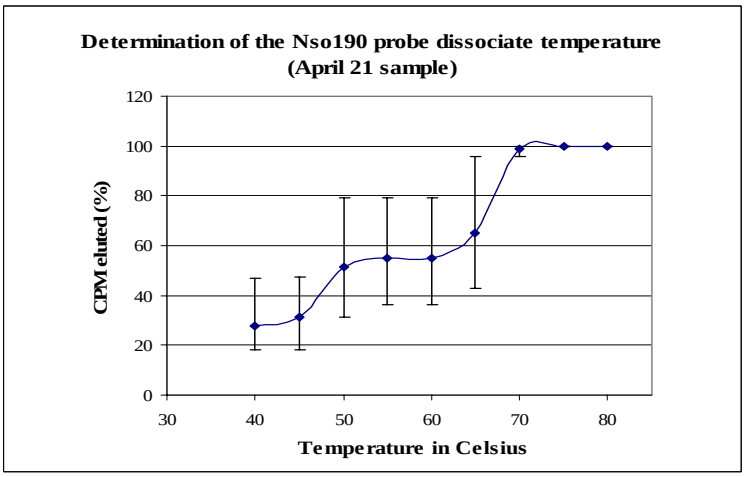
Determination of probe dissociation temperature. The probe dissociation temperature of Nso190 was determined by performing the experiment described above. The same experiment could not be done for Nsc128 since no results were obtained after the second exposure of the membrane. Only the membrane areas of the samples from March 15, April 21, and May 24 were used for determining probe dissociation temperature since these samples were the ones that showed well defined bands on the autoradiograph (Fig. 1). Figure 3 shows the probe dissociation temperature curves generated by plotting the accumulated radioactive count per minute (CPM) of the wash buffer against the temperature range used in the experiment. Figure 3a, 3b, and 3c show the curves of samples obtained from March 15, April 21, and May 24 respectively. These curves were generated by averaging the radioactive count of the triplicates of each sample. The approximate probe dissociation temperatures of Nso190 for the March 15, April 21, and May 24 samples were 51.9^{o}C, 49.4^{o}C, and 44.1^{o}C correspondingly.

DISCUSSION

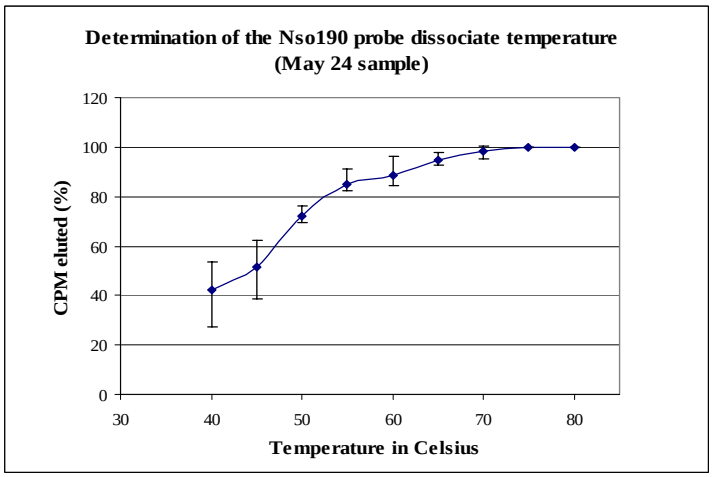
The main purpose of this experiment was to test for the presence of novel AOB in the bioreactor, which could not be detected by Nso190 but contributes to the continuous production of nitrite. The results of the experiment showed that the novel bacteria, namely *Nitrosococcus halophilus* and *N.oceani*, were not the bacteria that caused the discrepancy mentioned above. The autoradiograph of the membrane hybridized with Nsc128 probe showed no bands (Fig. 2a), which implies that the populations of *N.halophilus* and *N.oceani* in the bioreactor are not significant enough to produce large amount of nitrite and cause the discrepancy, or there are no *N.halophilus* and *N.oceani* in the bioreactor and therefore no 16S rRNA present to be detected by the probe. This is supported by the fact that



(a)



(b)



(c)

Figure 3. Probe dissociation temperature curves for Nso190. The probe dissociation temperature was determined by using samples from: (a) March 15, (b) April 21, and (c) May 24. Curves were generated by averaging the counts of the triplicates of each sample.

N.halophilus and *N.oceani* are the AOB in the γ-subdivision of the *Proteobacteria* that have only been found in marine environments (13). In addition, Watson (15), Carlucci and Strickland (3) estimated the *in situ* populations of *N.oceani* in enrichment cultures were low, and also the *in situ* nitrification rates were as low as 0.02 to 0.7 μmol liter^{-1} year^{-1}. Since these bacteria live in saline water, the SBR might not provide an appropriate environment for survival and therefore none of their 16S rRNA could be detected. RNA isolated from the ocean sample collected from Jericho Beach also did not show a positive result in the slot blot experiment even though *N.oceani* should be present in ocean surface water (11, 12, 14). This unexpected result might be due to insufficient *Nitrosococcus* cells collected from the ocean sample, or low populations of *N.halophilus* and *N.oceani* in the low salinity water at Jericho Beach.

Another probe, Nso190, was used to detect the presence of β subclass of *Proteobacteria* in the SBR. This probe was used so that the results obtained from the membrane hybridized with this probe could be compared with those observed from the membrane hybridized with Nsc128. Since no bands could be found on the autoradiograph of the Nsc128 hybridized membrane, no comparison could be made. Figure 2b shows the autoradiograph of the Nso190 hybridized membrane. Sample 2, which was collected on March 24, has no band shown on the autoradiograph. The result suggests that no β subclass of *Proteobacteria* was present when the sample was collected on March 24. However, the presence of bands of the March 15 and March 26 samples makes it unlikely that there is no β subclass of *Proteobacteria* in the SBR on March 24. Furthermore, the results obtained from agarose gel electrophoresis and absorption spectrum measurement (Table 2) indicate that the RNA concentration of the March 24 sample is the highest among all the samples. The absence of bands for sample 2 might be due to RNA degradation in the sample during the experiment.

Figure 2b also shows that the intensities of the bands are different between samples. The higher the intensity of the band, the more concentrated the detected β subclass *Proteobacteria* 16S rRNA was represented in the sample because more radioactive probes would bind to the sample. Since the area for sample 1 was removed, we can only compare the band intensities for the rest of the samples. Sample 4 and sample 5 should have the same band intensity as 8A and 8B respectively because 8A is from sample 4 while 8B belongs to sample 5. It appears that the concentration of β subclass *Proteobacteria* 16S rRNA increased from March 26 through May 24 as the autoradiograph shows an increase of band intensity from sample 3 through sample 4 (8A) to sample 5 (8B). This observation suggests that the composition of the SBR was changing and the amount of β subclass *Proteobacteria* in the bioreactor was increasing during that period. The faint bands for sample 6 and 8C show that β subclass *Proteobacteria* is present in the ocean sample collected from Jericho Beach, but the 16S rRNA of the bacteria in Jericho Beach is much less than that from the SBR. In addition, the similar band intensity of the triplicates for each sample shows the consistency of the results for each sample.

Figure 3 shows the probe dissociation temperature curves for Nso190 determined from different samples. Mobarry *et al.* (7) carried out a similar experiment and the temperature curves generated from their samples were sigmoidal. However, when comparing the results of this experiment to those of Mobarry's, only the curve generated from sample 1 (March 15) shows an expected sigmoidal shape. The plots of the other two samples have abnormal shapes. The abnormal shapes might be due to the mixed bacterial culture used in this experiment, while pure bacterial culture was analyzed in Mobarry's experiment. Nso190 can bind to different bacteria that belong to the same β subclass of *Proteobacteria.* For mixed bacterial culture, Nso190 might bind more efficiently to one bacterium's rRNA than the other due to the differences in rRNA sequence of different bacteria. Therefore, the probe dissociation temperature for different bacteria would be different. The two-step temperature curve in figure 3b might be a combination of two different probe dissociation temperature curves of two bacteria, with the midpoints of the two slopes representing the probe dissociation temperatures for the two bacteria.

The predicted dissociation temperature for the Nso190 (Table 1) was lower than the 62^{o}C probe dissociation temperature reported by Mobarry *et al* (7). The experimentally determined temperature ranges from 44.1^{o}C to 51.9^{o}C. The discrepancy could be due to different bacteria in the mixed culture having slightly different 16S rRNA sequences, leading to the lower probe dissociation temperature. The range of the dissociation temperature values also suggested that there was a continuous change of the bioreactor population.

The assumption that *N.halophilus* and *N.oceani* were the novel bacteria in the bioreactor cannot be supported by these results since the 16S rRNA of these bacteria could not be detected by the Nsc128 probe.

FUTURE EXPERIMENT

The failure to detect γ subclass bacteria in the slot blot hybridization experiment might not be due to the absence of the bacteria in the samples. Further experiments are needed to confirm the effectiveness of the probe since the sequence of the probe originally came from an unpublished source. In order to test the specificity of the probe for the γ subclass bacteria, a pure sample of the γ subclass bacteria should be used as the control instead of the ocean sample used in this experiment. Bands should be present if the probe is specifically designed for the γ subclass bacteria. In addition, one can also examine samples from another bioreactor that is operated under different conditions for comparison. The whole experiment should be repeated in order to verify the results.

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