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Characterization of the *Nitrobacter*-specific NIT3 and Nb1000 Probes and Their Use in the Detection of *Nitrobacter* Species in a Lab Scale Completely Stirred Tank Reactor System

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***Nitrobacter* species are responsible for the oxidation of nitrite to nitrate in the environment. Studies indicated that there is an inconsistent correlation between the amount of nitrifying activity in the bioreactor and the amount of nitrifying bacteria detected by the NIT3 and Nb1000 16S rRNA probes. This study investigates the explanation for the inconsistency, focusing on whether there are any sequence mismatches between the probes and the *Nitrobacter* 16S ribosomal RNA present in the environmental sample that preclude the detection of *Nitrobacter*. The samples used in this study were collected from a lab scale completely stirred tank reactor system seeded from the Kent wastewater treatment plant. The techniques employed to analyze samples included RNA isolation, radioactive labelling of 16S rRNA oligonucleotide probes, slot blot hybridization, and the determination of the dissociation temperature for both probes. Nucleic acid hybridization results suggested that *Nitrobacter* was present at a very low concentration in the stirred tank reactor, which was below the limit of detection using the radioactively labelled NIT3 and Nb1000 probes. Using RNA isolated from pure *Nitrobacter* strain, the experimentally measured dissociation temperature for the Nb1000 probe was 43ºC and the minimum dissociation temperature for the NIT3 probe was 54ºC. It was concluded that the amount of RNA immobilized onto the membrane should be increased by approximately 400-fold in order to obtain a detectable signal for *Nitrobacter*.**

Nitrification is a two-step aerobic process involving the oxidation of ammonium ion (NH_4^+) to nitrite (NO_2^-) and subsequent oxidation of nitrite to nitrate (NO_3^-). This process has great environmental implication as it is the major component of the nitrogen cycle found in ocean water, freshwater lakes, soils, aquaria, and sewage treatment systems (8). Nitrification is beneficial in the sewage treatment system because it helps to protect aquatic life and minimize oxygen depletion by reducing the amount of toxic ammonia in the environment. However, the process also causes pollution with the production of nitrogenous by-products such as nitric oxide, nitrate and nitrite ions. When these compounds are discharged into the receiving water, it has significant concerns related to the loss of fertilizers from soil and the creation of greenhouse effects (2, 11).

Bacteria belonging to the genera *Nitrosomonas*, *Nitrosococcus*, and *Nitrosospira* are examples of lithoautotrophs that are capable of converting ammonia to nitrite (6, 7). The second stage of nitrification, nitrite oxidation, is catalyzed by the nitrite-oxidizing bacteria. *Nitrobacter* and other related chemolithoautotrophic bacteria play important roles in nitrite oxidation. *Nitrobacter* is one of the well-investigated nitrite-oxidizing alpha *Proteobacteria*. They are Gram-negative, rod-shaped bacteria present in soil, freshwater, and the marine environments (2). *N. winogradskyi*, *N. hamburgensis* and *N. spp.* belong to the *Nitrobacter* genus (11).

Several techniques have been used in the past to enumerate and detect the nitrifying bacteria. A better understanding of the nitrifying composition of the sewage system would enable engineers to control the nitrification process in the wastewater treatment plants. The most probable number (MPN) technique involves the recording of the presence or absence of bacterial growth in several replicate dilutions of samples, while plating of pure cultures can also be used for quantifying nitrifiers (4). However, these cultivation-dependent techniques have disadvantages which include the time- and labour- intensive enumeration process and the difficulty in the identification of nitrifying organisms due to the slow growth of the bacteria and the small size of the colonies. Improved qualitative and quantitative techniques to study the nitrifying population, including the use of fluorescent *in situ* hybridization, PCR and *amoA* gene sequence analysis, and the ribosomal ribonucleic acid analysis, have been developed in the past 10 years. The 16S rRNA-targeted oligonucleotide probes have been specifically designed to detect the presence of a particular bacterial species in the environment (1, 4, 10, 14). The specificities of the probes targeted against

particular 16S rRNA sequences can be manipulated to understand the phylogeny and diversity of species within a microbial population.

However, recent findings indicated that the amount of activity of the nitrifying bacteria in bioreactors did not consistently correlate to the degree of binding by the 16S rRNA probes targeted for the nitrifying bacteria. This was found to be true for the *Nitrobacter* Nb1000 probe, which barely detected the presence of *Nitrobacter* species even when there was a high level of nitrate formation in the bioreactor (Rob Simm, personal communication, 2003). This inconsistency may be due to the sequence mismatch between the probe and the corresponding nitrifying organism in the reactor. The objective of this experiment was to determine if the discrepancy was due to the presence of novel strains of *Nitrobacter* present in the bioreactor, or a slightly different sequence between the probe and the 16S rRNA that resulted in a different dissociation temperature of the DNA/RNA hybrid. In addition, the construction of the dissociation temperature profiles for the probes would allow the optimization of the hybridization conditions.

MATERIALS AND METHODS

Sample Collection. Environmental samples were provided by Robert Simm and were collected from the R5 completely stirred tank lab scale reactor. The bioreactor was seeded from the Kent wastewater treatment plant in Agassiz, British Columbia, Canada. Five millilitres of samples, collected from the bioreactor on May 11, May 20, May 27, June 8, June 11, and June 14 of the year 2003, were stored in RNase-free COD vial. Each sample was centrifuged and the sludge pellet was stored at -80ºC until processing. RNA isolated from *Nitrobacter winogradskyi* (provided by Rob Simm) was used as a positive control as previous studies (11) reported that this species could be detected by the Nb1000 probe.

RNA Extraction. Total RNA was extracted from bacterial cells using the guanidinium thiocyanate-acidic phenol-chloroform (TRIzol® reagent) method as described in the Invitrogen™ Life Technologies manufacturer's protocol. For samples collected on May 11, 20, 27, and June 8, the pellet resulting from approximately 5 ml of the bioreactor's mixture was resuspended in 2 ml of TRIzol® reagent. The TRIzol® reagent is a monophasic solution of phenol and guanidine isothiocyanate and it disrupts cells while inactivating RNase activity. Only 1 ml of the mixture was used for RNA extraction. The remainder of the sample was stored at -80ºC for future experiment. For samples collected on June 11 and June 14, the whole pellet resulting from approximately 5 ml of bioreactor's bacterial mixture was resuspended in 1 ml of RNase-free diethylpyrocarbonate (DEPC)-treated water. The cells were centrifuged at 14,000 rpm at 4ºC for 4 min and the pellet was resuspended in 1 ml of TRIzol® reagent.

After the pellet was resuspended in TRIzol® reagent, the suspension was then transferred to a 2-ml screw cap microcentrifuge tube (Fisherband®). This was followed by the addition of 200 µl of chloroform to the suspension. In addition, glass beads of 0.1 mm diameter (BioSpec Products) were added until the microcentrifuge tube was filled to the top. Bacterial cells were broken mechanically by bead beating the suspension at room temperature, for three periods of 1 min each, with the Mini-BeadBeater-1™ (BioSpec Products) set to 4,800 rpm. RNA was precipitated from the upper aqueous layer with 0.5 ml of isopropanol and then washed with 1 ml of 75% ethanol. The pellet was air-dried briefly and re-dissolved in 100 µl of RNase-free water. RNA was quantified by measuring the sample absorbance at 260 nm and 280 nm with a spectrophotometer (Ultrospec®3000; Pharmacia Biotech). For RNA with a A_{260}/A_{280} ratio of less than 1.8, the concentration of RNA was calculated using the relationship [nucleic acid] (mg/ml) = 0.063 x A_{260} – 0.036 x A_{280} (12). An absorbance wave scan from A_{240} to A_{300} was obtained as a preliminary assessment of the quality of the isolated RNA.

Determination of the Quality of RNA using Gel Electrophoresis. The integrity of the extracted RNA was characterized by gel electrophoresis in 1X TAE buffer (40 mM Tris [pH 8.0], 1.14 ml of glacial acetic acid, 1 mM EDTA) with 1.2% agarose (13). The gels were stained with ethidium bromide and run at 70 V for about 1 hour. The gels were visualized and photographed under a UV transilluminator. Only samples with distinct 23S and 16S rRNA bands in an approximate ratio of 2-to-1 were used for hybridization (11).

Slot Blotting. Triplicates of each of the 6 samples (May 11, May 20, May 27, June 8, June 11, June 14), together with RNA isolated from pure *Nitrobacter* culture as a positive control, RNase-free water as a negative control, were analyzed by slot blotting for each individual probe. The RNA samples were denatured with 3 volumes of 2% glutaraldehyde in 50 mM sodium phosphate (pH 7.0) and incubated for 10 minutes at room temperature. They were then diluted with 1 µg/ml of poly(A) water so that 1 µg of RNA in a final volume of 200 µl was applied to each slot. The diluted RNA was immobilized onto 9 x 12 cm Zeta-Probe® nylon membrane (Bio-Rad Laboratories) with a 48-well slot blotter according to instructions given in the Bio-Dot SF blotting apparatus manual (Bio-Dot® SF Microfiltration Apparatus; Bio-Rad Laboratories). After blotting, the membrane was baked at 80ºC for 30 min and stored at room temperature before tube hybridization.

Oligonucleotide Probe Labelling. Probes NIT3 and Nb1000 were characterized previously to detect specifically members of the genus *Nitrobacter* (4, 11). The properties of the probes are indicated in Table 1. In this study, all probes were synthesized by the Nucleic Acid Protein Service Unit at the University of British Columbia. The probes were labelled with ^{32}P (Amersham Pharmacia Biotech) at the 5'end using the KinaseMax™ 5' end labelling kit (Ambion). The kinase reaction mixture contained 2 pmol of the probe, 8 pmol of [gamma-^{32}P]ATP (6000 Ci/mmol), 2 µl of 10X Kinase Buffer, and 1 µl of T4 Polynucleotide Kinase (10 U/µl). DNase-free water was added in order to obtain a final reaction volume of 20 µl. The reaction mixture was incubated at 37ºC for 1 hour. At the end, the labelled probes were separated from the unincorporated ATP with the QIAquick Nucleotide Removal Kit (QIAGEN). The probe labelling efficiency was checked using Cerenkov counting to quantify the amount of radioactivity in the labelled probes and the unincorporated [^{32}P]ATP that was washed off.

Table 1. 16S rRNA phylogenetic probes used to detect *Nitrobacter* species in this study. The characteristics of the NIT3 probe was stated in Coskuner's article (reference 4) and the probe dissociation temperature for the Nb1000 probe was experimentally determined by Mobarry *et. al.* (reference 11).

Probe	16S rRNA position	Sequence (5' to 3')	Probe dissociation temperature (ºC)	Targeted group
NIT3	1035-1048	CCT GTG CTC CAT GCT CCG	46	*Nitrobacter spp.*
Nb1000	1000-1012	TGC GAC CGG TCA TGG	42	*Nitrobacter spp.*

Tube Hybridization. Identical membranes (3 cm x 6 cm) with immobilized RNA were prepared for hybridization with the NIT3 and Nb1000 probes. The two sets of membrane were put in separate pre-warmed (40ºC) roller bottles for each specific probe. In the pre-hybridization step, approximately 12 ml of pre-warmed (40ºC) hybridization buffer (0.9 M NaCl, 50mM $NaPO_4$, 5 mM EDTA, 10X Denhardt's solution (0.2% Bovine serum albumin, 0.2% Ficol 400, 0.2% Polyvinylpyrrolidone dissolved in distilled water) 0.5% SDS, 0.5 mg of poly (A) per ml) was added to each tube and incubated in the hybridization incubator (Model 308; Lab-line Instrument) at 40ºC for 3 hours. In the hybridization step, approximately 2 x 10^7 CPM of radioactively ^{32}P-end-labelled NIT3 and Nb1000 probe was added to each bottle with the membrane and was incubated in the hybridization oven overnight at 40ºC. The temperature of the hybridization oven was lowered to 35ºC on the following day and the membranes were incubated at this temperature for an additional 3 hours. After that, the hybridization buffer was replaced with approximately 60 ml of wash buffer (1X SSC (0.15 M NaCl, 0.015 M sodium citrate), with 1% SDS) and the membranes were washed twice at 35ºC for 30 min each. At the end of the washing step, the membranes were allowed to air dry at room temperature. Film autoradiographs were recorded with an intensifier screen in a developing cassette for a period of 12 hours to 23 days.

Determination of the Dissociation Temperature (T_d) of NIT3 and Nb1000

From the autoradiographs, only samples with acceptable radioactive hybridization signal were chosen for the probe T_d determination experiment. The corresponding bands on the membrane were excised into separate strips and each strip was placed in a 0.65 ml polypropylene microcentrifuge tube containing 500 µl of wash buffer (1X SSC (0.15 M NaCl, 0.015 M sodium citrate), with 1% SDS), which was pre-warmed at the starting temperature of 35°C in the themocycler (Techne PHC-3). Replicates were subsequently washed for 30 min each at 5°C temperature increments ranging from 35°C to 65°C. After each wash, the membrane was transferred to new wash buffer incubated at elevated temperature. The wash buffer from the previous temperature was transferred to a scintillation vial containing 1.25 ml of scintillation cocktail (Ultima Gold, Packard Instruments). This was used for the quantification of radioactivity by liquid scintillation counting with the scintillation counter (Beckman LS6000TA). The probe's dissociation profile was established by plotting the cumulative % of radioactivity eluted versus temperature.

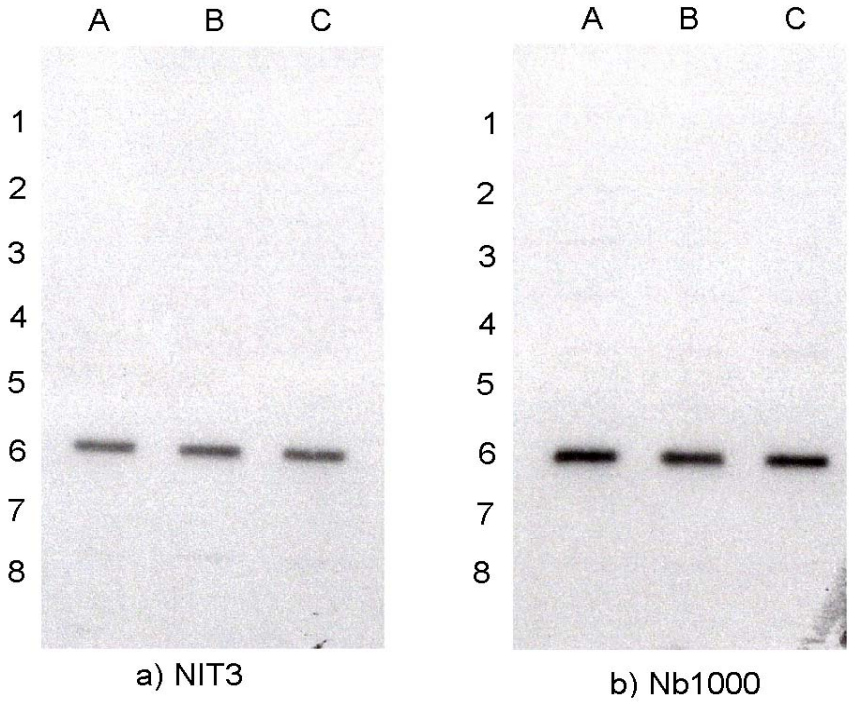

Figure 1. Autoradiograph (overnight exposure) showing the specificity of the (a) NIT3 and (b) Nb1000 probes for *Nitrobacter*. The RNA extracted from the bioreactor's mixed liquor collected on the indicated dates were blotted in the slots in the following arrangement: row 1: May 20, 2003; row 2: May 27, 2003; row 3: June 8, 2003; row 4: June 11, 2003; row 5: June 14, 2003; row 6: RNA from *Nitrobacter* pure culture (positive control); row 7: RNase-free water (negative control); row 8: May 11, 2003. A, B, and C are replicates of the RNA sample.

RESULTS

Slot blot hybridization. The specificity of the NIT3 and Nb1000 probes for *Nitrobacter* was demonstrated in Fig. 1a and 1b. In the overnight-exposed autoradiograph, a strong positive signal was detected in the RNA isolated from *Nitrobacter* pure culture, in which both NIT3 and Nb1000 probes hybridized to the triplicate RNA samples. The labelling efficiency for both NIT3 and Nb1000 probes indicated that there was about 10% of labelled probe with

respect to unlabelled probe. The positive signal obtained from the Nb1000 probe was stronger than the NIT3 probe as the bands corresponding to the amount of radioactively labelled probe bound to RNA were more intense for the Nb1000 probe. The hybridization signal for both NIT3 and Nb1000 probes on all the bioreactor samples was too low to be detected.

Only samples resulting from the positive control had a detectable amount of radioactivity so the bands were excised for the probe's T_d determination experiment. The remainder of the hybridized membrane was further exposed to 23 days in order to determine whether longer exposure would produce a stronger signal. Figures 2a and 2b show that, upon longer exposure, both NIT3 and Nb1000 probes detected the presence of *Nitrobacter* in all samples. No signal was detected on the negative control as predicted. In general, the band intensity for samples collected on different dates displayed a similar trend on the autoradiographs for the NIT3 and Nb1000 probes. For the NIT3 probe, samples collected on May 11 and June 11, 2004 indicated a stronger signal, while sample collected on May 20 indicated a very weak signal. For the Nb1000 probe, samples collected on June 11, 2004 produced the strongest hybridization signal. The intensity of the bands resulting from the May 11 samples (Fig. 2a, row 8) was approximately the same as the intensity of the bands from the pure *Nitrobacter RNA* detected by NIT3 (Fig. 1a, row 6). As a result, it could be estimated that bands with this intensity corresponded to approximately 649 CPM (analysis not shown). This was determined by the addition of the radioactive counts, corresponding to the amount of radioactive probes washed off at each temperature. In general, the radioactive signals of the sample bands and the background were stronger for the Nb1000 probe than with the NIT3 probe.

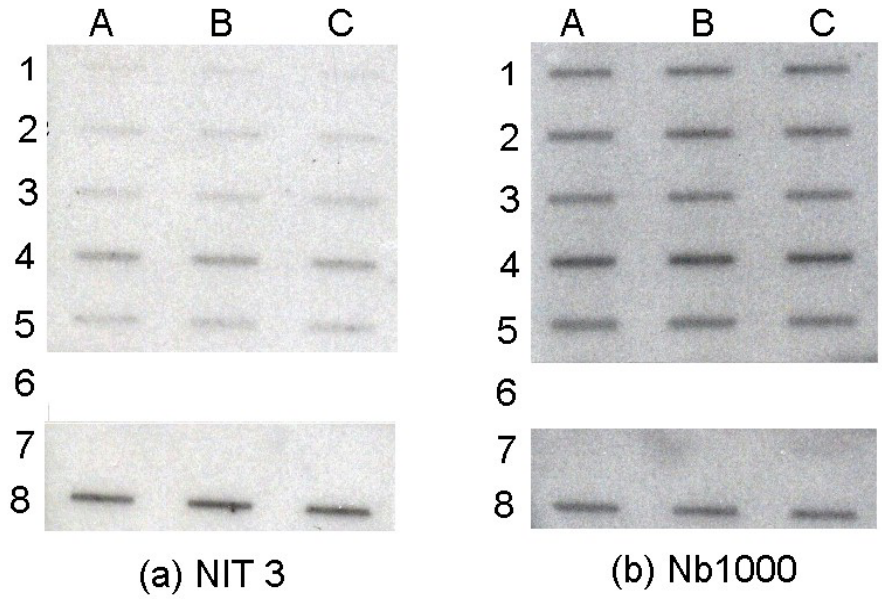

Figure 2. Autoradiograph (23-day exposure) showing the specificity of the (a) NIT3 and (b) Nb1000 probes for *Nitrobacter*. The RNA extracted from the bioreactor's mixed liquor collected on different dates were blotted in the slots in the following arrangement: row 1: May 20, 2003; row 2: May 27, 2003; row 3: June 8, 2003; row 4: June 11, 2003; row 5: June 14, 2003; row 6: RNA from *Nitrobacter* pure culture (positive control) – bands were excised from the membrane; row 7: RNase-free water (negative control); row 8: May 11, 2003.
A, B, and C are replicates of the RNA sample.

Determination of the Dissociation Temperature for the NIT3 and Nb1000 Probes. RNA isolated from the *Nitrobacter* pure culture was used for the probe dissociation temperature determination experiment. The dissociation temperature is defined as the temperature at which 50% of the bound probe is dissociated from the rRNA-DNA hybrid (1). It was measured by the amount of probes eluted over an increasing temperature wash series from 35ºC to 65ºC. Figures 3a and 3b show the probe dissociation temperature profiles of the NIT3 and Nb1000 probes. The results for the triplicate samples were consistent, with less than 5% standard deviation. For the NIT3 probe, the amount of probe dissociated increased slowly from 16% to 51% as temperature was increased from 35ºC

to 55ºC, and the rate of probe dissociation continued to increase to 1.5 times when temperature was increased from 55ºC to 65ºC. There was no saturation point to indicate when all the probes had been washed off. Therefore, it could only be estimated that the minimum dissociation temperature (T_d) for the NIT3 probe should be above 54ºC (Fig. 3a). For the Nb1000 probe, the dissociation profile resembles a part of a sigmoid plot and the maximum amount of bound probe reached a saturation point after 55ºC (Fig. 3b). The measured dissociation temperature (T_d) for the Nb1000 probe was 43ºC.

DISCUSSION

Hybridization Experiment. RNA isolated from the environmental samples were immobilized on nylon membrane and hybridized with NIT3 and Nb1000 probes separately. After overnight exposure of the membrane, no radioactive signal was detected on the autoradiograph. However, a longer exposure of 23 days and the presence of bands indicated that *Nitrobacter* was present in all samples collected on different dates. Thus, the selection of the detection system is an important parameter when considering the sensitivity of the hybridization assay. The reasoning is that a longer exposure would enable a weaker radioactive signal to be detected on the film. It should be noted that although a longer exposure of the film would allow more radioactive signal to be detected, the radioactive ^{32}P would also decay at a specific rate, which would affect the amount of radioactivity being quantified in the T_d determination experiment. The failure to detect any radioactive signal on the autoradiograph could be attributed to several factors. First, the concentration of *Nitrobacter* was low in the environmental sample. Thus, there was only a small composition of *Nitrobacter* RNA bound to the radioactively labelled NIT3 and Nb1000 probes among the total RNA being isolated. This would have resulted in radioactive signal below the detection limit. Second, different species of *Nitrobacter* present in the bioreactor may have 16S ribosomal RNA with slightly different nucleotide sequences, preventing optimal hybridization to the probes. Third, some unlabelled probes may still have been present after the QIAgen clean-up washing step and bound to the *Nitrobacter* RNA, reducing the amount of RNA substrate available to bind to the labelled probes.

The results revealed that *Nitrobacter* bacteria were present at very low concentration in the samples used in this study. Therefore, they might not be the dominant species responsible for nitrite oxidation in the R5 stirred tank reactor seeded from a wastewater treatment plant. In order to confirm this, we could use different probes against various nitrifying species and determine which organisms were present at high concentration. The experimental findings agree with the results from earlier studies, which used rDNA library analysis and denaturing gradient gel electrophoresis to demonstrate that the *Nitrospira*-like species was the dominant bacteria for the oxidation of nitrite to nitrate in freshwater and marine aquaria (5, 8, 9). In addition, Daims *et al.* examined the population of nitrite-oxidizers in a sequencing batch biofilm reactor by fluorescent *in situ* hybridization and digital image analysis and concluded that there were higher cell numbers for *Nitrospira* than for *Nitrobacter* (6).

Although the same amount (1 µg) of RNA was immobilized onto the Zeta-Probe membrane for each sample, the intensity of the signals detected by the NIT3 and Nb1000 probes were different. Autoradiographic results showed that the Nb1000 probe showed better binding to *Nitrobacter* than did the NIT3 probe, as indicated by the darker bands on the Nb1000 autoradiograph (Fig. 1 and 2). The sample bands, together with the background signal on the autoradiograph for the Nb1000 probe, were darker than the autoradiograph for the NIT3 probe. The higher intensity on one autoradiograph may be due to the presence of more non-specific binding with the Nb1000 probe or incomplete washing of the unlabelled probes after the hybridization step. Another explanation is that the Nb1000 probe may detect additional *Nitrobacter* species that either contain nucleotide mismatches or the presence of a novel species that cannot be detect by the NIT3 probe. As a result, more bacteria was bound to the Nb1000 probes, giving a stronger signal. The hybridization of different probes was performed in different roller tubes. Other than this inconsistency, the probe labelling efficiency, hybridization and detection conditions were constant.

It was also observed that the band intensity pattern of different samples varied slightly between the probes. For example, the May 20 sample gave a very weak signal when hybridized with the NIT3 probe in comparison to the May 27 sample. However, the signals for the May 20 and May 27 samples were similar in the hybridization with Nb1000 probe (Fig. 2). A plausible explanation is that the NIT3 and Nb1000 probes detect different 16S rRNA regions of a particular nitrifying organism. In the May 20 sample, the Nb1000 probe may have better binding to a novel nitrifying species that cannot be detected by the NIT3 probe.

The replicates within each sample in this study yielded consistent and reproducible results. As expected, bands corresponding to samples collected on the same date have the same intensity (Fig. 2). Environmental samples collected on different dates established different profiles on the autoradiograph. There were some variations in the representation of *Nitrobacter RNA* in samples collected during the period from May 11 to June 14. For example, samples collected on June 11 showed darker bands, indicating a higher composition of *Nitrobacter* in the bioreactor

on that day. The change in the composition of the nitrite-oxidizing bacteria *Nitrobacter* may be due to condition changes in the bioreactor. For example, a change in the ammonia concentration or the amount of dissolved oxygen in the bioreactor may affect the growth of the *Nitrobacter* population. Interestingly, high concentration of nitrite and oxygen also favoured the growth of *Nitrobacter spp.* compared to *Nitrospira*-like bacteria, while low nitrite and nitrite and oxygen concentrations favoured the growth of the *Nitrospira*-like bacteria (5).

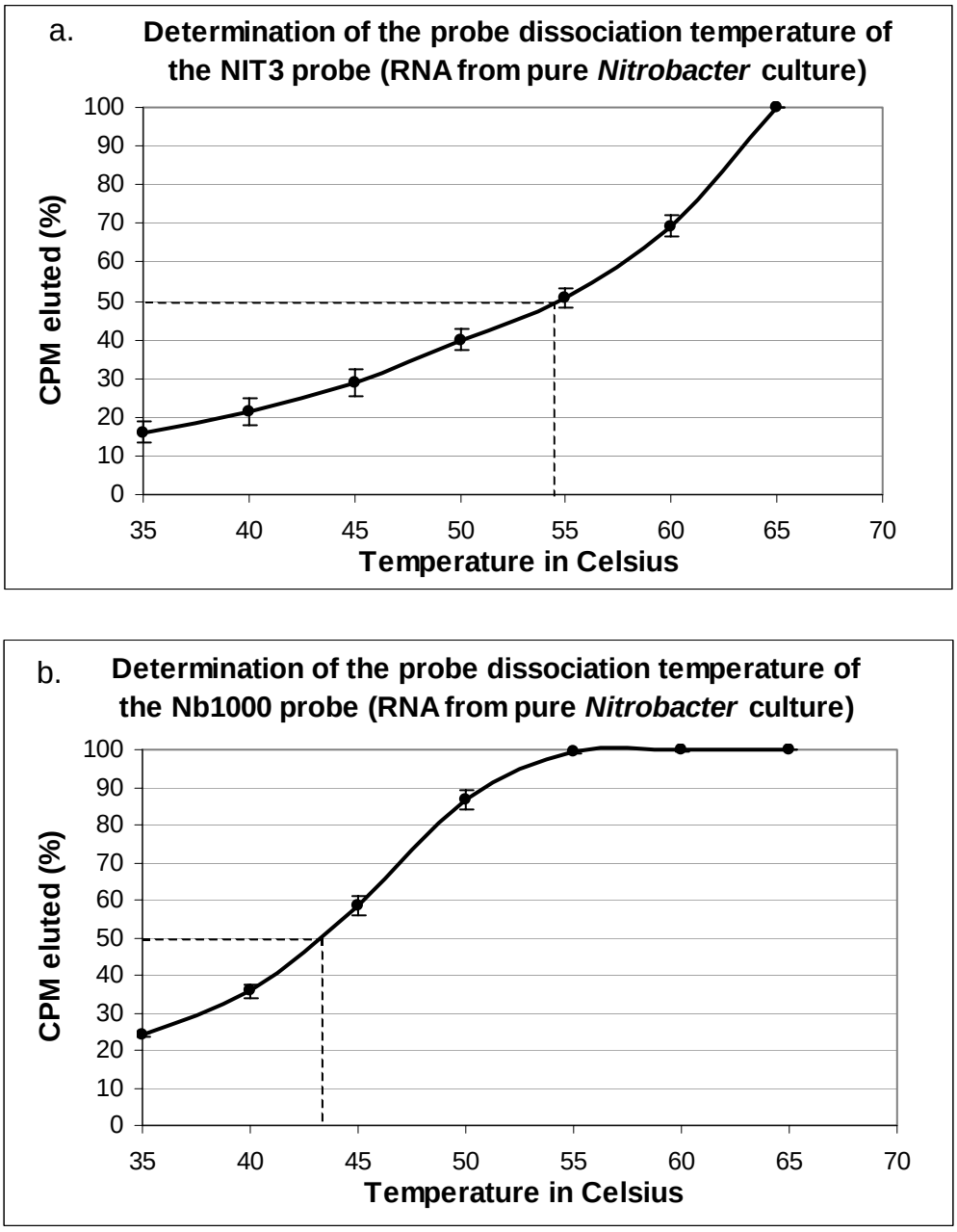

Figure 3. Probe dissociation temperature profiles for the (a) NIT3 and (b) Nb1000 probes over a temperature range from 35ºC to 65ºC. The amount of probe dissociation is expressed as the cumulative percentage of radioactive counts per minute (CPM) eluted from the hybrid. The average value among the triplicates is plotted, with y-error bars indicating the standard deviation among the triplicates. The temperature of dissociation for each probe is denoted by the dotted vertical line.

Probe Dissociation Temperature Determination Experiment. As indicated in Fig. 3a and 4a, the NIT3 probe displayed a different dissociation profile compared to the Nb1000 probe and other oligonucleotide probes used in the study by Mobarry (11). The continuous increase of the amount of radioactive probes being washed off from 35°C to 65°C without a point of levelling off was unexpected. Detailed examination of the dissociation temperature profile indicated the possibility of the existence of two separate curves with different slopes. Our experimentally measured minimum T_d for the NIT3 probe, 54˚C, disagreed with Coskuner's T_d of 46°C (4). However, it was close to the melting temperature (55ºC) calculated using oligonucleotide calculator available on the internet. The calculator uses the properties of the nucleotides and the equation, $T_d = 4N_{G+C} + 2 N_{A+T}$, in which N_{G+C} and N_{A+T} correspond to the contents of A, T, C, and G nucleotides. The following differences in parameters in the hybridization reaction may result in the difference of the dissociation temperature. First, RNA isolated from *Nitrobacter winogradskyi* pure culture was used in T_d determination experiment in this study, while a different *Nitrobacter* species might have been used in Coskuner's experiment. Moreover, the hybridization conditions, including the cationic concentration in the hybridization buffer, the length of the hybridization, and the washing temperature were different between both studies (4).

The experimentally measured T_d, 43ºC, agreed with the T_d presented by Mobarry, 42ºC. This result was possibly more reliable and comparable because Mobarry also measured the T_d profile of the Nb1000 probe on *Nitrobacter winogradskyi* and the hybridization conditions were similar to the parameters used in this study (11).

For both NIT3 and Nb1000 probes, the failure to obtain a symmetrical sigmoidal curve was attributed to the fact that the initial starting hybridization temperature was at 40°C, which may have reduced the amount of probe hybridized to the RNA. As a result, almost a quarter of the probe had been washed off at 35°C. This can influence the shape of the curve, which represents the accumulative radioactivity being washed off at each specific temperature. If this experiment is to be repeated in the future, the pre-hybridization, the hybridization temperature and the first washing temperature should be set at around 25°C to allow the probe to be washed off gradually at the beginning.

The weak radioactive signal corresponding to the amount of *Nitrobacter* bound to both NIT3 and Nb1000 probes limited the ability to study the diversity of *Nitrobacter* species in the R5 complete stirred tank reactor. However, results from the positive control in the hybridization experiment proved that both NIT3 and Nb1000 16S rRNA probes could be used to detect *Nitrobacter*. It was also concluded that the microbial nitrifying composition in the R5 reactor was different from the systems used in previous studies. Further optimization of the NIT3 probe is necessary. If both probes can be used to identify *Nitrobacter* in the environmental systems, it will be very helpful for researchers to study the populations of nitrification.

FUTURE EXPERIMENTS

The experiment could be improved in order to amplify the amount of detectable radioactive signal. This could be achieved by increasing the amount of RNA loaded on the membrane for the slot blot experiment, adding more radioactive ^{32}P to the probes, or exposing the autoradiograph for an additional 1-2 days before the T_d determination experiment.

The autoradiographs for the positive control samples could be used to estimate the amount of RNA samples needed to obtain a detectable signal for *Nitrobacter* in future experiments. We could correlate the following information obtained from the 1-day and 23-day exposure of the autoradiographs for the NIT3 probe: (i) the half life of ^{32}P is 14.3 days (ii) the band intensity of the May 11 sample on the 23-day exposed autoradiograph for the NIT3 probe (Fig. 2a, row 8) is approximately the same as the band intensity of the positive control band on the 1-day exposed autoradiograph for the NIT3 probe (Fig. 1a, row 6) (iii) the band intensity of the May 20 sample, the band showing the weakest signal, is approximately 1/30 of the band intensity of the May 11 sample on the 23-day exposed autoradiograph for the NIT3 probe. It was estimated that at least a 400-fold increase in the amount of total bacterial RNA should be used in future experiments.

A more accurate way to estimate the amount of RNA needed from the samples can be achieved by loading a series of known concentration of RNA isolated from the *Nitrobacter* pure culture. For example, 0.1 µg, 0.5 µg, 1 µg, 10 µg, and 100 ug of the positive control RNA can be loaded. The positive control, together with the sample RNA, will be immobilized and hybridized under the same conditions, and the membrane should be exposed for the same amount of time. Therefore, we can directly compare the intensity of the bands in a more reliable fashion. The use of the Phosphor Imager to quantify the intensity of the bands would be beneficial. We can also optimize the hybridization parameters, such as the hybridization buffer, time of hybridization and wash temperature, for each probe. After this step, we can use the optimal T_d in the PCR, hybridization, and sequence analysis experiments to study the specificity of the probes against the *Nitrobacter* species present in the reactor. Furthermore, we can use

the probes to detect the *Nitrobacter* species in other systems. The observation that *Nitrospira*, instead of the traditionally identified *Nitrobacter*, is the dominant nitrite-oxidizers has not been studied in details. The conditions in the bioreactor can be varied in order to examine the relationship between these two types of nitrite-oxidizers.

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