Application of Molecular Techniques to the Characterization of a Nitrifying Bioaugmentation Culture

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(ABSTRACT)

Nitrification is the biological process whereby ammonia is converted first to nitrite by ammonia-oxidizing bacteria, and then the nitrite is subsequently converted to nitrate by nitrite-oxidizing bacteria. Ammonia and nitrite levels are closely monitored during treatment of wastewater due to their toxicity to other biological processes. Sybron Chemicals, Inc., is a company that manufactures a nitrifying bioaugmentation culture (1010N) that is used to enhance the naturally occurring levels of biological nitrification. The microbial population of the 1010N product has been examined using a combination of conventional bacteriological methods and modern molecular techniques, with the goal of developing nucleic acid probes that can be used to detect the product in an environmental sample. Small regions of the 16S rRNA genes of the bacteria in 1010N (and two new nitrifying enrichment cultures) were amplified via the polymerase chain reaction (PCR) and analyzed via temperature gradient gel electrophoresis (TGGE). TGGE is a procedure that allows for separation and visualization of individual PCR products that are the same size, based on differences in their sequence. Two of the predominant PCR products in 1010N were purified from the TGGE gel matrix, reamplified via PCR, and sequenced to allow for phylogenetic analysis and nucleic acid probe design. Coincidentally, two strains (NS500-9 and MPN2) that had been isolated from the 1010N mixed consortium and grown in pure culture were found, via TGGE, to have identical 16S rRNA sequences to the PCR products under investigation. Nearly the

full-length 16S rRNA genes from these two organisms were PCR amplified, cloned, and sequenced in order to provide a basis for more accurate phylogenetic analysis. The two dominant organisms in the 1010N product, NS500-9 and MPN2, were thereby found to be most closely related to *Nitrosomonas* and *Nitrobacter*, respectively, in the existing database. Using the nucleic acid sequences of the cloned DNA, organism-specific DNA probes were designed for both NS500-9 and MPN2. Unfortunately, difficulties were encountered in using the probes to monitor 1010N activity levels via quantitative dot blot hybridizations (rRNA-DNA). Therefore, efforts were redirected to using the TGGE semi-quantitatively with an internal PCR standard (Brüggeman, *et al*., 2000) to estimate original cell numbers of 1010N within a mixed consortium. This method was not applicable to our system due to substantial preferential binding of the primers to template other than the standard. Samples from a laboratory-scale bioreactor, bioaugmented with 1010N, were used in an attempt to correlate an increase in activity with a detectable shift in population via TGGE. No detectable shift in population was detected in these samples even though the system exhibited increased levels of nitrification. Therefore, the sensitivity of the TGGE system was also examined by determining the limits of detection when 1010N was present in activated sludge. In both whole cell spiking experiments and genomic DNA spiking experiments, it was found that 1010N must be present at a level of at least 5% of the total population in order to be detected. While this provides some information about microbial populations, in order to evaluate the biological activity of a system, nucleic acid probes should be used in a rRNA based study.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Nitrification

The recycling of nitrogen in the environment is essential to life's existence. Nitrification, a multi-step biological process, is an important part of the nitrogen cycle and also an integral component of water treatment systems. The overall reaction that defines nitrification is the conversion of ammonia to nitrate. Nitrate is a more oxidized form of nitrogen that can be utilized by plants and other bacteria. The reaction occurs as shown in Figure 1. The first step is the oxidation of ammonia to nitrite, with hydroxylamine formed as an intermediate. This reaction is carried out by chemolithoautotrophic ammonia-oxidizing bacteria (AOB). The nitrite is then converted to nitrate by nitrite-oxidizing bacteria (NOB).

In the late 19th century, scientists began to acknowledge and isolate nitrifying bacteria (Frankland and Frankland, 1890, Winogradsky, 1890). Since then, much work has been done in order to establish phylogenetic relationships between the various distinct groups. Significant progress has been made in the last ten to fifteen years, as powerful molecular tools have provided the field with unprecedented insights. On a general level, the nitrifying bacteria are currently considered to be one coherent group, the family *Nitrobacteriaceae* (Watson *et al*., 1989). By definition, this requires the ability to grow lithotrophically through the oxidation of ammonia to nitrite, or nitrite to nitrate. To date, an organism that is capable of directly converting ammonia to nitrate has not been discovered. As a result, this large group of bacteria has been traditionally divided into two groups, ammonia-oxidizing bacteria vs. nitrite-oxidizing bacteria.

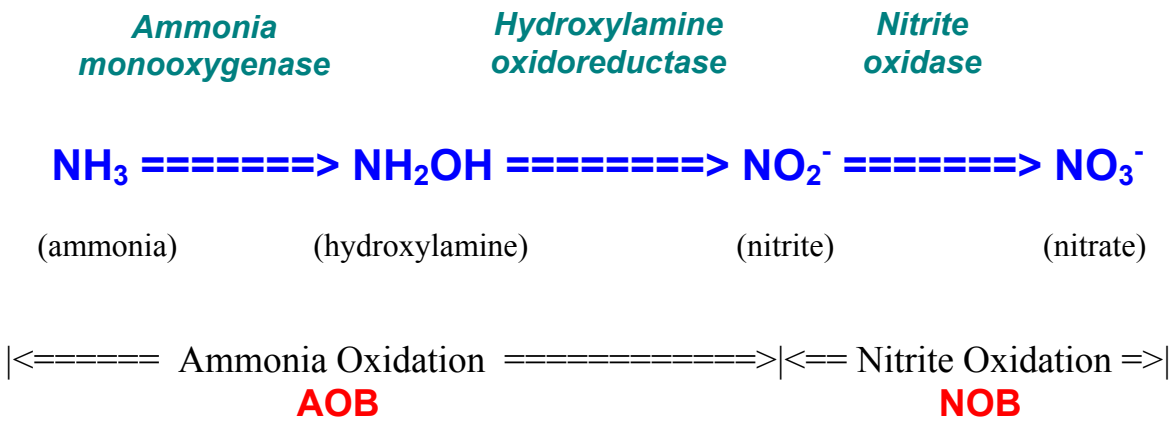


Figure 1: The process of nitrification, see text for details.

However, the phylogenetic relationship of the organisms within these two groups is considerably more complex.

Studies done by Carl Woese and colleagues (1987, 1984, 1985) have shown that all characterized nitrifying bacteria are proteobacteria, a large group that has been traditionally generalized as the purple photosynthetic bacteria. The nitrifying bacteria are dispersed throughout the alpha, beta, gamma, and delta subdivisions of the proteobacteria. While the framework for this system of classification is 16S rRNA sequence alignments, structural and physiological characteristics are also important contributing factors. The alpha subdivision includes the well-studied nitrite-oxidizing genus *Nitrobacter*, but no other statistically significant aquatic nitrifiers. Nearly all of the ammonia-oxidizing bacteria belong to the beta subdivision, which currently excludes any nitrite-oxidizers. The genera named in this subdivision are *Nitrosomonas* (reportedly the most prevalent), *Nitrosococcus*, *Nitrosospira*, *Nitrosolobus*, and *Nitrosovibrio*. The gamma subdivision is unique in that it is possible to find species of both ammonia-oxidizing bacteria (*Nitrosococcus oceanus*) and nitrite-oxidizing bacteria (*Nitrococcus mobilis*). The delta subdivision includes the more recently discovered nitrite-oxidizing genera *Nitrospira* and *Nitrospina*. Based on their protein composition, they are only very distantly related to the nitrite-oxidizers in the alpha and gamma subdivisions (Teske, 1994). Recent studies are showing the importance and ubiquity of these species in the environment, overturning a long-held assumption that *Nitrobacter* holds the primary role in the oxidation of nitrite (Bouchez *et al*., 2000, Wagner *et al*.,1996 and 1998).

Regardless of the genus and species, the nitrifying bacteria are notoriously difficult to isolate and maintain. One problem is that they are very sensitive to changes in

environmental conditions. For example, they are greatly affected by swings in temperature and pH, and are also sensitive to chemical inhibition. In addition, the growth rates of both ammonia- and nitrite-oxidizing bacteria are extremely low. Generation times vary from 8 hours for *Nitrosomonas* to 10 hours for *Nitrobacter* to 60 hours for *Nitrosospira* (Prosser, 1986). With so many difficulties it is surprising that such a group of bacteria could be advantageously employed in a wastewater treatment system. Nonetheless, biological nitrification has been established as an important and effective method of reducing levels of ammonia in wastewater treatment facilities.

The removal of ammonia is important for various reasons, such as its toxicity. At sufficiently high levels, ammonia is more harmful to fish and other aquatic life than nitrate (Painter, 1986). In addition, it creates an oxygen demand in receiving waters that ultimately results in lower dissolved oxygen concentrations. Ammonia also contributes to eutrophication in lakes. These environmental consequences demonstrate the need for nitrification as a wastewater treatment process, and motivate researchers to explore ways to create a more refined and manageable system.

Many wastewater treatment facilities that incorporate nitrification into their treatment process rely on bioaugmentation to increase overall levels of microbial activity. Bioaugmentation is the addition of selected microbes to a system in order to enhance the existing population, resulting in improved water quality. Due to the dynamic nature of wastewater treatment plants, the biological population is constantly changing. This is especially evident with changing seasons as a result of the temperature sensitivity of nitrifying bacteria. Typically, a significant reduction in nitrification levels is observed during the fall and winter months. In the spring, a bioaugmentation product may be

added to quickly shift the population towards a more desirable composition with regard to activity and efficiency. It has been shown that the product must be added on a regular basis in order for the bioaugmentation to be successful, mostly because of the temporary stability of the newly introduced strains (Boon *et al*., 2000).

The effectiveness of bioaugmentation is difficult to study in the field because it would require a wastewater plant to run two identical but isolated systems side by side, which in most locations is not a realistic option. Various studies (Bouchez *et al*., 2000, Boon *et al*., 2000) have been conducted in an attempt to demonstrate either the benefit or failure of this approach. In this thesis project, the bacterial composition of a nitrifying bioaugmentation product (1010N) manufactured by Sybron Chemicals, Inc. (Salem, VA), has been examined using a combination of culture-based and molecular techniques. This culture, although used by several wastewater treatment facilities, had not been previously characterized. Identification of the predominant bacteria in this product, along with the ability to detect its presence in a system, should have future implications with regard to improvement of nitrification efficiency and the marketability of the product.

The 16S rRNA gene

It has been repeatedly demonstrated that only a small fraction of bacteria existing in various environments has been identified, primarily due to the presence of non-culturable organisms (Wagner *et al*., 1993, Jannasch and Jones, 1959). Specifically, in seawater it has been estimated that less than 0.1% of the bacterial population is culturable (Kogure *et al*., 1980). In comparison, soil has an equally low percentage of 0.3% (Torsvik *et al*., 1990), whereas in activated sludge the percentage is anywhere from 1-

15% (Wagner *et al*., 1993 and 1994). This disturbing reality was originally brought to attention by the "great plate count anomaly", as it was called by Staley and Konopka (1985). They repeatedly found that direct microscopic counts substantially exceeded viable-cell counts, resulting in the meager estimates stated above. The cause, as discussed in a review by Amann *et al*. (1995), is a combination of two possibilities: (i) identified species for which the existing cultivation conditions are insufficient or which have entered a nonculturable state, or (ii) unknown species that have not yet been cultured and have unknown growth requirements.

The nitrifying bacteria are just one example of many microorganisms that have been difficult to culture as a result of complex growth requirements. For example, the nitrifying bacteria require a wide range of chemicals in order to enable growth, and unfortunately do not grow well on solid media (Prosser, 1986). Once growth requirements are determined, another difficulty encountered is the ability to isolate a unique species such that it can be studied in pure culture. A variety of bacteria can be found in nature growing as aggregated microcolonies within thick sludge flocs (diameter up to 50 μm), including the ammonia- and nitrite-oxidizing bacteria which have been shown to grow in close contact (Wagner *et al*., 1994, Mobarry *et al*., 1996, Juretschko *et al*., 1998). This type of spatial arrangement substantially decreases the probability of complete isolation and growth in pure culture.

In order to circumvent the problems associated with cultivation, scientists have turned to rRNA. These molecules are found in all organisms, and their sequence has changed slowly but randomly over time in such a manner that the variations can be used to measure phylogenetic relationships. The nature of the molecule is useful in that

certain regions are highly conserved across the primary kingdoms, whereas others show substantial variance down to the level of species (Woese, 1987). While earlier studies focused on the 5S rRNA (Stahl *et al*., 1984 and 1985), the 16S rRNA quickly became the molecule of interest as sequencing capabilities improved. The average size of a 16S rRNA gene is 1500 bp, which is sufficient for preliminary phylogenetic analysis (Woese, 1987).

The highly defined characteristics of the 16S rRNA have been exploited in order to compensate for the difficulties with culture-based methods. To obtain preliminary information, bacteria no longer need to be cultured in the lab. Instead, they can be taken directly from their natural environment and analyzed. As previously mentioned, there are regions of the 16S rDNA that are conserved among mostly all eubacteria. These regions conveniently serve as universal binding sites for oligonucleotides. This provides an excellent tool for studying mixed bacterial consortia. A total DNA extraction can be performed to give a mixed pool of chromosomal DNA. A pair of universal primers is then chosen such that the polymerase chain reaction (PCR) can be used to amplify through a highly variable region of the 16S rRNA gene. The region used in this project and other studies (Muyzer *et al*., 1993) is the variable region V3. The result of this PCR reaction is a mixed pool of products that are equal in size, but which exhibit species-specific sequence variation. A number of molecular approaches can then subsequently be used to analyze and manipulate the PCR products, thereby allowing for the characterization of an unknown bacterial population.

Temperature Gradient Gel Electrophoresis (TGGE) and Denaturing Gradient Gel Electrophoresis (DGGE)

Both temperature and denaturing gradient gel electrophoresis work on the same basic principle. When denaturing conditions are applied to double-stranded DNA molecules during electrophoresis, they will denature in discreet sequence-specific regions known as melting domains (Figure 2A). As part of the molecule becomes denatured, its migration through the gel is significantly inhibited. A pool of PCR products amplified from the 16S rDNA of a mixed bacterial consortium will exhibit variation in sequence, making this type of electrophoresis a useful tool in examining the bacterial composition.

Numerous studies employ TGGE in order to study complex bacterial populations (Felske *et al*., 1998, Eichner *et al*., 1999). TGGE utilizes temperature as the variable denaturant. The concentration of the chemical denaturant, urea, is uniform throughout the gel and therefore a constant factor. As electrophoresis is carried out the temperature of the running buffer is slowly and uniformly increased. The result is the ability to separate fragments based on differences in sequence rather than size (Figure 2B), as is done with a standard polyacrylamide or agarose gel. Denaturing gradient gel electrophoresis (DGGE) is also a commonly used technique (Muyzer *et al*., 1993, Kowalchuk *et al*., 1997, Brüggemann *et al*., 2000) and is only slightly different from TGGE. Rather than using a temperature gradient, a chemical gradient is employed. The temperature is held constant, but the gel itself is poured to give an increasing concentration of urea along the same direction as electrophoresis. When a critical concentration of urea is reached, the melting domains will denature and result in a

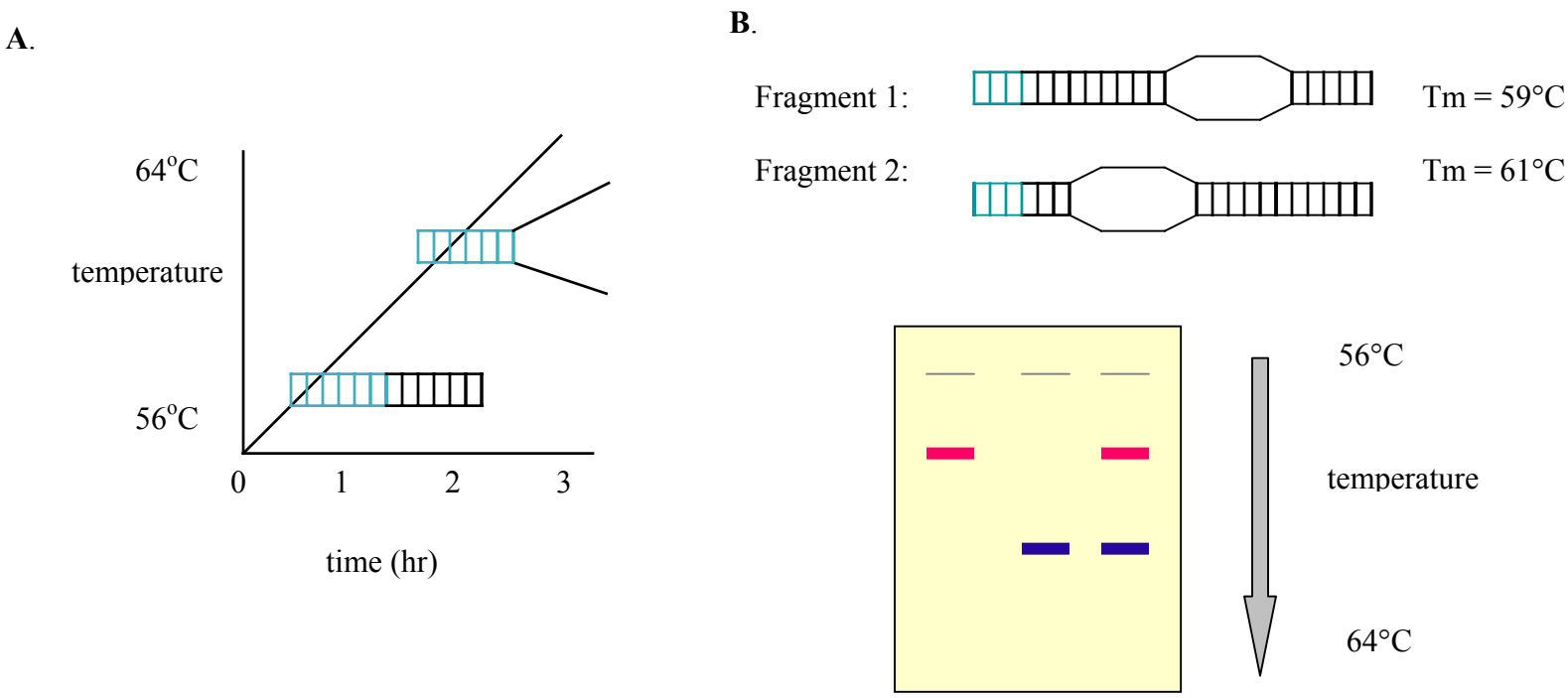


Figure 2: Cartoon models of the separation of PCR amplified fragments via TGGE

Panel A: TTGE is a technique that exploits the melting properties of DNA. Over time with increasing temperature, a double stranded DNA molecule will denature in discreet sequence specific regions known as melting domains. In the case where there are two fragments of the same size that differ in sequence, it is possible to separate them using TGGE.
Panel B: In the hypothetical situation illustrated, Fragment 1 has a sequence which results in a melting domain with a melting temperature (Tm) of 59°C. Fragment 2, which is exactly the same size, has a slightly different sequence that results in a melting domain with a Tm of 61°C. A sample containing a mixture of these two fragments is loaded into the gel and the temperature increased during electrophoresis. The result is that as the temperature reaches 59°C, the melting domain of fragment 1 denatures and its migration is inhibited. Similarly, as the temperature reaches 61°C, the melting domain of fragment 2 denatures and its migration is inhibited. This results in a complete separation of multiple fragments based only on their difference in sequence. In order to ensure that the molecule doesn't completely denature and run through the gel as two single-stranded molecules, the

separation of fragments based on sequence. With both DGGE and TGGE, it is necessary to design the primers in a way that will result in the formation of a "GC-clamp" at one end of the fragment. This clamp is simply a long string of GC-repeats that gives one section of the fragment a high melting point, prohibiting it from completely denaturing into two single-stranded molecules during electrophoresis.

While both the TGGE and DGGE methods are widely accepted, TGGE was chosen for this research project. As previously mentioned, the variable factor in DGGE is the concentration of the chemical denaturant in the gel. This necessitates a precise technique with regard to the physical preparation of the gel in order to obtain consistent results. In contrast, because the variable factor in TGGE is temperature, the gel is prepared with a uniform concentration of urea. The simplified nature of the TGGE gel, along with the fact that the temperature is controlled electronically by the Dcode Universal Mutation Detection System (Bio-Rad) and therefore reliably consistent between runs, provides a basis for reproducible data.

Denaturing electrophoresis quickly provides qualitative information regarding the diversity of the bacterial composition of a mixed culture, but to identify the bacterial components requires further manipulation. Theoretically, each distinct band is indicative of one unique species. A potential problem that must be given consideration is multiple fragments having similar mobility. In order to demonstrate that a band of interest only contains one species of PCR product, it may be excised and the nucleotide sequence analyzed. This sequence data can then be used for the design of nucleic acid probes as discussed below.

Nucleic Acid Probes

Just as the conserved regions of the 16S rRNA gene provide universal binding sites for primers, the variable regions provide sites to which a carefully designed nucleic acid probe can anneal in a highly specific manner. These probes can be designed such that they are targeted to large taxonomic units, or conversely, one particular species. This is done by aligning the rDNA sequences, identifying sequence idiosyncrasies, designing the complementary nucleic acid probe, and then optimizing the experimental conditions to achieve the desired probe specificity (Amann *et al*., 1995). Once designed, molecular probes can be used with a variety of techniques in order to study a mixed population. For example, it is possible to determine the relative abundance of a particular 16S rRNA in comparison with the total 16S rRNA with quantitative dot blot hybridizations. This provides information regarding the percent of physiological activity that can be attributed to a particular organism, or group of organisms, within a given system. It cannot be directly correlated to cell numbers, as the ribosomal content is related to growth rate. As a result, the rRNA content of cells of the same strain may vary by orders of magnitude depending on their physiological state. (Delong *et al*., 1989, Kemp *et al*., 1993). Dot blot and Southern hybridizations can also be performed with rDNA in order to obtain qualitative information about the presence/absence of an organism of interest. Again, exact information regarding cell numbers is not obtained with this method due to inherent bias in both DNA extraction and PCR methods, along with the fact that the copy number of the rRNA gene can range from 1 to 10 copies (Amann *et al*, 1995). In order to get more accurate cell counts, recent studies (Wagner *et al*., 1998) have employed fluorescent *in situ* hybridization (FISH). This method uses fluorescent-labeled probes to

detect specific rRNA sequences within whole cells, as they occur in their natural environment. By using fluorescent labels having different emission and excitation wavelengths, it is possible to simultaneously apply probes specific to different organisms. With the use of a confocal scanning microscope, it is possible to obtain a 3-dimensional image that clearly illustrates the spatial distribution of the organisms of interest.

In this study, TGGE was initially used to determine the complexity of the bacterial composition of the 1010N product. The two predominant isolates (NS500-9 and MPN2), as identified through TGGE analysis, were also coincidentally the two strains that had been isolated in pure culture from 1010N. This facilitated the construct of plasmids encoding nearly the full-length 16S rRNA genes from these two strains. Using nucleotide sequence information provided by both the TGGE gel and the cloned 16S rRNA genes, nucleic acid probes specific to NS500-9 and MPN2 were successfully designed and utilized following some of the strategies outlined in Figure 3.

CHAPTER TWO

MATERIALS AND METHODS

Isolation and maintenance of nitrifying bacteria from 1010N

Two strains of nitrifying bacteria were isolated in pure culture from the 1010N culture by Sybron, using the Most Probable Number (MPN) technique. These two strains were designated NS500-9 (an ammonia-oxidizing bacterium), and MPN2 (a nitrite-oxidizing bacterium). Throughout the duration of this study, both organisms were maintained as actively growing cultures in the following medium (for a 1 liter volume): 0.2 g $MgSO_4 \cdot 7H_2O$, 0.02 g $CaCl_2 \cdot 2H_2O$, 0.087 g K_2HPO_4, 2.52 g EPPS Buffer (Sigma), 1 ml Solution B (100 mg chelated iron/100 ml ddH_2O), 1 ml Solution C (10 ml of 100 mg $Na_2MoO_4 \cdot 2H_2O$/100 ml ddH_2O, 17.2 ml of 100 mg $MnSO_4 \cdot H_2O$/100 ml ddH_2O, 0.4 ml of 100 mg $CoCl_2 \cdot 6H_2O$/100 ml ddH_2O, 10 ml of 100 mg $ZnSO_4 \cdot 7H_2O$/100 ml ddH_2O, to a total volume of 100 ml with ddH_2O), 0.5 ml Solution D (500 mg phenol red (Fisher Scientific) /100 ml ddH_2O). For the ammonia-oxidizing bacterium, NS500-9, 1.32g ammonium sulfate was also added to the media. For the nitrite-oxidizing bacteria, MPN2, 3.0 ml 4% sodium nitrite and 0.5 N-Pep (1.4 g trypticase peptone, 1.4 g bactopeptone, and 0.2 g yeast extract in 60 ml dH_2O, filter sterilized) were added to the media.

The media was initially inoculated with 3 ml of viable log phase culture provided by Sybron, after which the culture was monitored on a weekly basis for pH levels. The pH was maintained between 7.0 and 7.4 using 20% potassium carbonate. In addition,

levels of nitrite and nitrate were also monitored on a weekly basis. Once a week, 3.0 ml 4% sodium nitrite was added to the MPN2 culture, and 3 ml of a 2.38 g/10 ml stock of ammonium sulfate was added to the NS500-9 culture. Every 4-5 weeks, cultures were transferred to fresh media.

Genomic DNA purification

Total genomic DNA extractions of pure culture isolates, the 1010N product, other bioaugmentation cultures, and activated sludge were carried out based on a protocol used by Ma (1999). A 700 µl aliquot of each sample was centrifuged (Eppendorf Centrifuge 5415C, Westbury, NY) for 10 min at 13,000 rpm, and then resuspended in 700 µl of TE buffer (10 mM Tris and 1 mM EDTA pH 8.0). The cells were then placed in a 2 ml polypropylene conical tube containing 0.7 g of glass beads (diameter 0.1mm, Biospec Products, Bartlesville, OK), 500 µl of phenol (pH 8.0, Fisher Scientific, Pittsburgh, PA), and 50 µl of 10% sodium dodecyl sulfate (SDS). Cell disruption was carried out in three, one-minute intervals at 5,000 rpm using a Mini Beadbeater (Biospec Products). The sample was centrifuged at 13,000 rpm for 1 minute to collect the glass beads, after which the aqueous phase was removed. The DNA was then purified by three sequential extractions with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and a final extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The nucleic acid was precipitated overnight with one-tenth volume of 5 M sodium chloride and 2 volumes of ethanol. The DNA sample was collected by centrifugation, dried, and resuspended in 100 µl TE buffer. Samples were quantified

using a Spectronic Genesys 5 spectrophotometer (Spectronic Instruments, Rochester, NY).

Primer Design

See Table 1 for the sequences of the primers used in this study, and Figure 4 for their relative sites of annealing on the 16S rRNA gene.

PCR amplification of a portion of the 16S rRNA gene for TGGE analysis

A DNA fragment of approximately 200 base pairs was PCR-amplified from a total genomic DNA sample of either mixed populations or pure cultures, using universal eubacterial oligonucleotide primers 341f-GC and 534r targeting the variable V3 region of the16S rRNA gene (Muyzer *et al*, 1993). Each 100 µl PCR reaction contained a final concentration of the following reagents: 100 ng of purified genomic DNA, 200 ng of each of the primers (Sigma-Genosys, The Woodlands, TX), 200µM dNTPs (Promega, Madison, WI), 1X *Taq*2000 Reaction Buffer (10 mM Tris-HCl pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin) (Stratagene, La Jolla, CA), 1.3 mM $MgSO_4$ (Fisher, Springfield, NJ), and 2.5 units *Taq*2000 Polymerase (Stratagene, La Jolla, CA). A Sprint thermal cycler (Hybaid, Middlesex, UK) was used to carry out a version of "touchdown PCR" (Don *et al*, 1991) as follows: one cycle: 94°C for 3 minutes; 5 cycles: 94°C for one minute, 65°C for 1 minute, and 72°C for two minutes; the annealing temperature was then decreased by 1°C every cycle until a touchdown at 55°C, at which temperature an additional 10 cycles were completed. A final primer extension was carried out at 72°C for 10 minutes. The PCR products were then analyzed by electrophoresis on a 0.8%

Table 1: 16S rRNA gene sequencing primers and probes

Primer Name	**Primer Sequence** (5' to 3')	**Reference**
27f	AGAGTTTGATCMTGGCTCAG	Lane, 1991
341f	CCTACGGGAGGCAGCAG	Muyzer et al., 1993
341f-GC	CGCCCGCCGCGCGCGGCGGGCGGGG CGGGGGCGCGGGGGGCCGACGGGAG GCAGCAG	Muyzer et al., 1993
534r	ATTACCGCGGCTGCTGG	Muyzer et al., 1993
700f	CACTGGCCCGATACTGAC	This study
907r	CCGTCAATTCMTTTRAGTTT	Lane, 1991
907f	AAACTCAAAGGAATTGACG	This study
1492r	TACGGYTACCTTGTTACGACTT	Lane, 1991
Probe Name	**Probe Sequence**	
EUB338	GCTGCCTCCCGTAGGAGT	Stahl and Amann, 1991
NS5445	ATTATTCGTTGCAATCCTTTCTTTCCG	This study
MPN996	AAGAGAGGGTCACATCT	This study

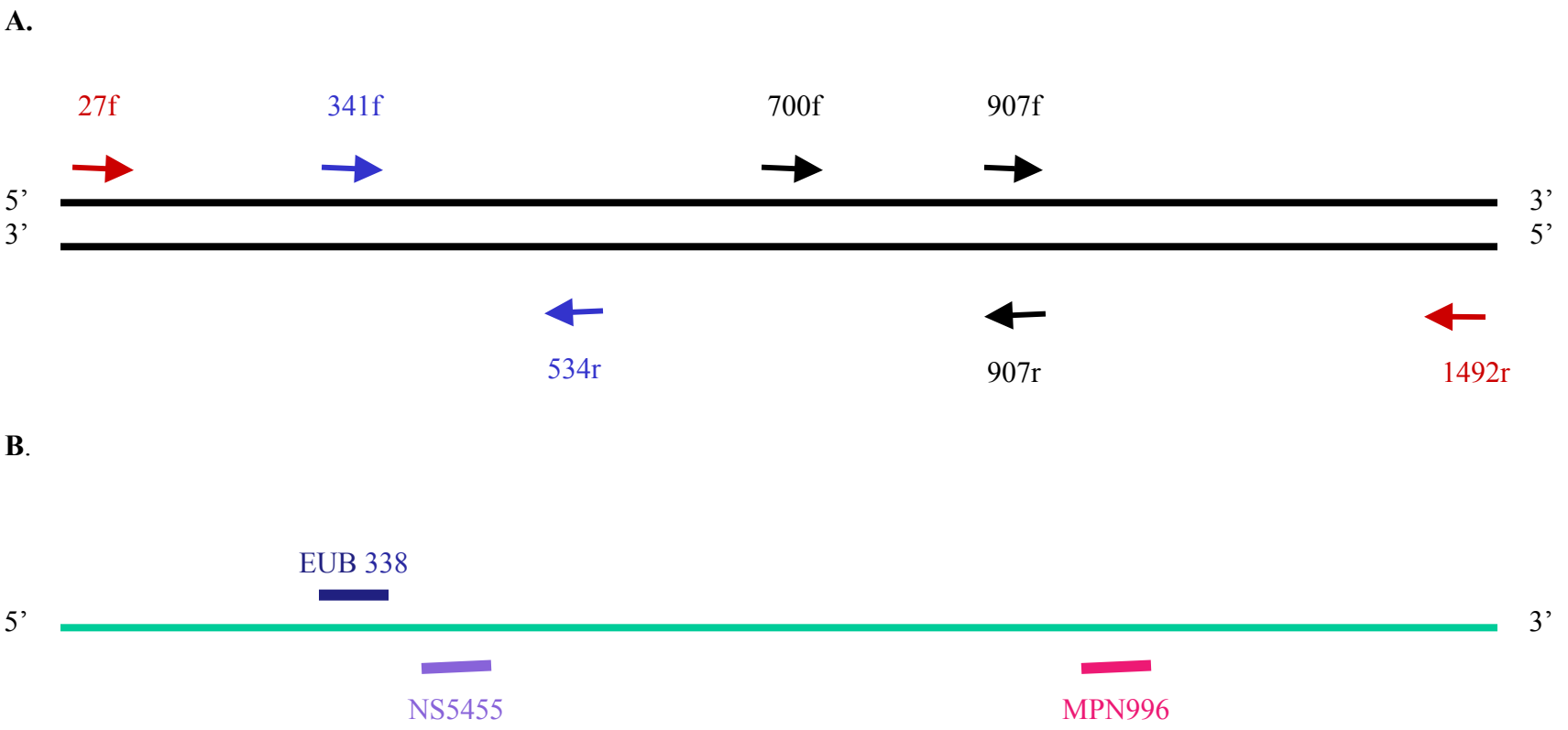


Figure 4. **Relative annealing sites of primers and probes used in this study**.
Panel A: Model of 16S rRNA gene with position of primers (arrows point towards the 3’ end of primer sequence).
Panel B: Model of 16S rRNA with position of nucleic acid probes (sequences of probes are complimentary to the rRNA sequence).

agarose gel (Bio-Rad, Hurcules, CA) in 1X TAE buffer (50X TAE is 242 g Tris, 57.1 g glacial acetic acid, 100 ml 0.5 M EDTA pH 8.0, in a total volume of 1 liter), and quantified by comparison to a Precision Mass Standard (Bio-Rad). In some cases, where the precise concentration was essential to a subsequent experiment, the samples were purified with the Qiagen PCR Purification kit (Qiagen Inc., Valencia, CA), and then quantified using a spectrophotometer.

Temperature gradient gel electrophoresis (TGGE)

A 10 μl volume of each PCR-amplified sample (approximately 500-750 ng of DNA) was applied with 2X Gel Loading Dye (Bio-Rad) to a 10% (wt/vol) polyacrylamide, 7 M urea gel, and subjected to electrophoresis using the Universal Dcode Mutation Detection System (Bio-Rad) for 6.5 hours at a constant voltage of 100 V in 1.25X TAE running buffer. Electrophoresis was carried out over a temperature range of 55°C – 63.5°C, with an increase of 1.3°C/h. The gel was then incubated for 40 minutes in 1X TAE containing Vistra Green (diluted 1:10,000) (Amersham Pharmacia Biotech, Piscataway, NJ), and scanned with a Storm® 860 PhosphorImager (Molecular Dynamics, Sunnyvale, CA), using blue-excited fluorescence at 800V.

Recovery and sequence analysis of bands from TGGE gels

The bands visualized on the TGGE gels from the pure cultures NS500-9 and MPN-2, and the two bands of corresponding mobility from 1010N, were excised from the gel and extracted as described in Maniatis *et al*, 1989. A sterile spatula was used to excise the bands of interest, which were then crushed and incubated overnight on a

shaker (250 rpm) at 37°C in 2 volumes of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1mM EDTA, pH 8.0). The polyacrylamide matrix was collected by centrifugation at 12,000 rpm for 2 minutes. The supernatant was removed, and the solid phase was rinsed by vortexing with one-half volume elution buffer. The solid phase was again collected by centrifugation, and the two supernatants combined. A 2-hour ethanol precipitation at -20°C followed by a 10-minute centrifugation at 13,000 rpm was then carried out in order to recover the purified nucleic acid. The DNA was reamplified using primers 341f (without the GC clamp) and 534r in a PCR reaction prepared as previously described. Amplification was carried out as follows: 1 cycle: 93°C for 3 minutes; 30 cycles: 93°C for 1 minute, 63°C for 1 minute, and 72°C for 2 minutes; 1 cycle: 72°C for 10 minutes. The PCR reactions were prepared for sequencing using the PCR Purification kit (Qiagen, Valencia, CA). All samples were sequenced on both strands with the same primers used for PCR. Nucleotide sequencing reactions were completed at the University of Iowa DNA Sequencing facility (Iowa City, IA).

Cloning of 16S rRNA genes

In order to obtain more accurate phylogenetic information regarding the pure culture isolates, the nearly full-length 16S rRNA genes of pure cultures NS500-9 and MPN2 were amplified using universal eubacterial primers 27f and 1492r (Lane, 1991). PCR reactions were prepared as previously described and carried out as follows: 1 cycle: 93°C for 3 minutes; 30 cycles: 93°C for 1 minute, 50°C for 1 minute, and 72°C for 2 minutes; 1 cycle: 72°C for 10 minutes. Following amplification, 3 μl of each PCR reaction was electrophoresed on a 0.8% agarose gel along with a mass ladder (Bio-Rad)

for quantification. Ligation of the PCR products into a cloning vector was completed using the pGEM-T vector system (Promega) as recommended by the manufacturer. Ligation reactions were used to transform *Escherichia coli* strain DH5α and plated on Luria-Bertani (LB) agar containing 100 μg/ml ampicillin, 1 mM IPTG, and X-gal (40 μl from a stock concentration of 20 mg/ml in dimethylformamide). Transformants were grown in LB broth containing 100 μg/ml ampicillin, and plasmid purifications were subsequently performed. The plasmid DNA was screened for the presence of the 1500 bp insert by restriction endonuclease digestion with *Sal*I (New England Biolabs, Beverly, MA), followed by electrophoresis of the linearized DNA through a 0.8% agarose gel. The plasmid constructs obtained were designated pNS15 for the NS500-9 insert and pMP15 for the MPN2 insert.

Nucleotide sequence analysis of 16S rRNA genes

The pNS15 and pMP15 DNA was prepared for nucleotide sequencing using the QIAprep Miniprep kit (Qiagen). The entire fragment of each 16S rRNA gene cloned was sequenced on both strands. In addition, another strand from a second isolate was sequenced to verify the correct sequence. Universal vector primers T7 and M13, along with internal primers 341f, 700f, 907f, 907r, and 534r (Table 1 and Figure 4A) were used to obtain complete sequence data. All sequencing reactions were carried out at the Virginia Tech DNA Sequencing facility. The 16S rRNA gene sequence for both NS500-9 and MPN2 was assembled using the SeqMan and MegAlign programs in Lasergene99.

Probe Design

The 16S rDNA sequences of both NS500-9 and MPN2 were aligned with published sequences of the 16S rDNA sequences of other ammonia- and nitrite-oxidizing bacteria, retrieved from the public database, including GenBank, European Molecular Biology Laboratory (EMBL), and the DNA Databank of Japan (DDBJ), with the BLAST program at the National Center for Biotechnology Information (NCBI). Regions of unique sequence in NS500-9 and MPN2 in comparison to the most closely related organisms currently in the database were more closely analyzed. The Check_Probe program supported by the Ribosomal Database Project (RDP) was used in order to design a probe that was complementary to the 16S rRNA of the target organisms but had mismatches to non-target organisms. Probe sequence was also checked for secondary structure and self-dimer formation using the PrimerSelect program in Lasergene99. The probe sequences that were designed and used in hybridization studies (NS5455 and MPN996) can be found in Table 1, and their relative sites of annealing are shown in Figure 4B.

Isolation of rRNA

All solutions used in protocols involving rRNA isolation were treated with 0.1% DEPC (diethyl pyrocarbonate) in order to inhibit RNases. DEPC is added to the solution, which is then autoclaved after a 12 hour incubation at 37°C. All surface areas and equipment were treated with RNase Away (Molecular BioProducts, San Deigo, CA) in order to remove RNase contamination.

In order to recover rRNA from bacterial cells, samples were prepared as previously described for genomic DNA, but with the following modifications. An equal volume of hot phenol (65°C), pH 5.0, was used in the initial beadbeating step. Three subsequent extractions were carried out using an equal volume of hot phenol-chloroform-isoamyl alcohol (25:24:1), followed by one extraction with an equal volume of chloroform-isoamyl alcohol (24:1). The nucleic acid was precipitated overnight in two volumes of ethanol and one-tenth volume 5 M NaCl. The rRNA was collected by centrifugation, dried to remove residual ethanol, and resuspended in DEPC-H_2O. Immediately following resuspension, the rRNA was analyzed via electrophoresis on a 0.8% agarose gel along with a rRNA standard (Boehringer Mannheim), and loaded onto a nylon solid support as described below for further analysis.

Dot Blot Hybridization Procedures with probe EUB338

DNA fragments of approximately 1500 bp were PCR-amplified from *E.coli* DH5α chromosomal DNA using primers 27f and 1492r and used along with universal eubacterial probe, EUB338 (Stahl and Amann, 1991) in order to determine the critical parameters for the dot blot hybridization detection system.. A ten-fold serial dilution of the DNA was prepared, with the concentration ranging from 1 μg to 10 ng. The diluted DNA series was then applied to Hybond N+, a positively charged nylon membrane (Amersham Pharmacia Biotech) using the Bio-dot Microfiltration apparatus (Bio-Rad) as recommended by the manufacturers. The DNA was subsequently fixed to the membrane by baking for 2 hours at 80°C. The membrane was prehybridized for 1 hour at 60°C in 5X SSC (20X SSC is composed of 0.3 M sodium citrate and 3 M NaCl), 0.1% (w/v)

SDS, 0.5% (w/v) dextran sulphate (MW 500,000), and a 20-fold dilution of liquid block (Amersham Pharmacia Biotech). Hybridization was carried out overnight at room temperature with EUB338 (100 ng), pre-labeled with a 5'-fluorescein tag (Genosys). A series of stringent washes were carried out at 58°C as follows: 3 x 10 minutes with 2X SSC, 0.1% SDS; and 3 x 10 minutes with 0.1X SSC, 0.1% SDS (Ma, 1999). Signal was then detected using the ECF signal amplification module (Amersham Pharmacia Biotech) as directed by the manufacturer. After a 1-hour "blocking step", the membrane was incubated with an anti-fluorescein alkaline phosphatase conjugate diluted 500-fold in freshly prepared 0.5% (w/v) bovine serum albumin in buffer A (0.3M NaCl, 0.1M Tris-HCl pH 7.5) at room temperature for 1 hour. The excess conjugate was removed from the membrane by three 10-minute washes in 0.3% Tween 20 (Fisher Scientific) in buffer A, after which the membrane was briefly washed in buffer A. The membrane was then placed in a clear plastic bag (supplied with the ECF signal amplification module), followed by the addition of the detection reagent (~25μl/cm^2). The probe-bound alkaline phosphatase cleaves the ECF substrate, yielding a highly fluorescent product. Fluorescence was observed using the Storm® 860 PhosphorImager (Molecular Dynamics) set at 800V with blue-excited fluorescence.

Once the limits of detection were determined with DNA, membranes loaded with rRNA samples were analyzed using this chemifluorescent system. The EUB338 probe was used to detect rRNA extracted directly from the 1010N product, rRNA extracted from an actively growing culture of *E.coli* DH5α, and commercially available rRNA standards (Boehringer Mannheim) as a control. rRNA samples were denatured by the addition of 3 volumes of 2% gluturaldehyde. Immediately following, serial dilutions of

the denatured rRNA were prepared in 1μg/ml poly(A) (Sigma) in water, and then applied to the membrane in various concentrations, ranging from 100 ng to 10 μg. A positive control membrane loaded with dilutions of DNA target (100 ng – 1 μg) was included in every RNA trial to ensure the detection system was functioning properly. Once the rRNA loaded membranes had been prepared, some of the remaining RNA sample was analyzed on a 0.8% agarose gel in order to ensure that degradation had not occurred during the loading process**.**

Optimization of Hybridization Conditions for probes NS5455 and MPN996

The experimental temperature of dissociation (Td) is the temperature at which one half of the bound probe is released from the probe-target duplex (Mobarry et al, 1996). The Td the two novel probes designed in this study (NS5455 and MPN996) was determined using DNA as the target nucleic acid. The target DNA for each probe was PCR-amplified from the 16S rRNA gene of the corresponding clone (pNS15 or pMP15). Primers 341f and 907r were used to generate a fragment of approximately 550 bp as target DNA for NS5455; and primers 907f and 1492r were used to generate a fragment of nearly 600 bp as target for MPN996. Target DNA from *E.coli* strain DH5α was also amplified for a negative control, using the same sets of primers. PCR reactions were prepared and carried out as described in the "cloning" section, using approximately 3 ng of plasmid DNA (clones pNS15 and pMP15) for template and an annealing temperature of 53°C. All reactions were analyzed via electrophoresis on an 0.8% agarose gel. DNA samples were then applied to a nylon membrane as previously described, resulting in six identical blots for each probe. All blots were handled as discussed under "dot blotting".

Each probe (100 ng) was added to its respective hybridization tube for an overnight hybridization at room temperature. The stringent wash steps were carried out over a range of temperatures: 30°C - 60°C (in 4°C increments) for NS5455, and 32°C - 52°C (in 3°C increments) for MPN996. Following the wash steps, the membranes were treated as previously described in order to detect signal (protocol outlined in Figure 5). The membranes were scanned using the Storm® 860 PhosphoImager, and the intensity of the signal was quantified using ImageQuant® software (Molecular Dynamics). The resulting fluorescence emission values were used to calculate the percentage of probe annealed at each temperature increment for repeated trials. Data for each trial was used to construct a dissociation curve for the two probes. The set of data that resulted in the highest Td was used to designate the experimental Td of the probe.

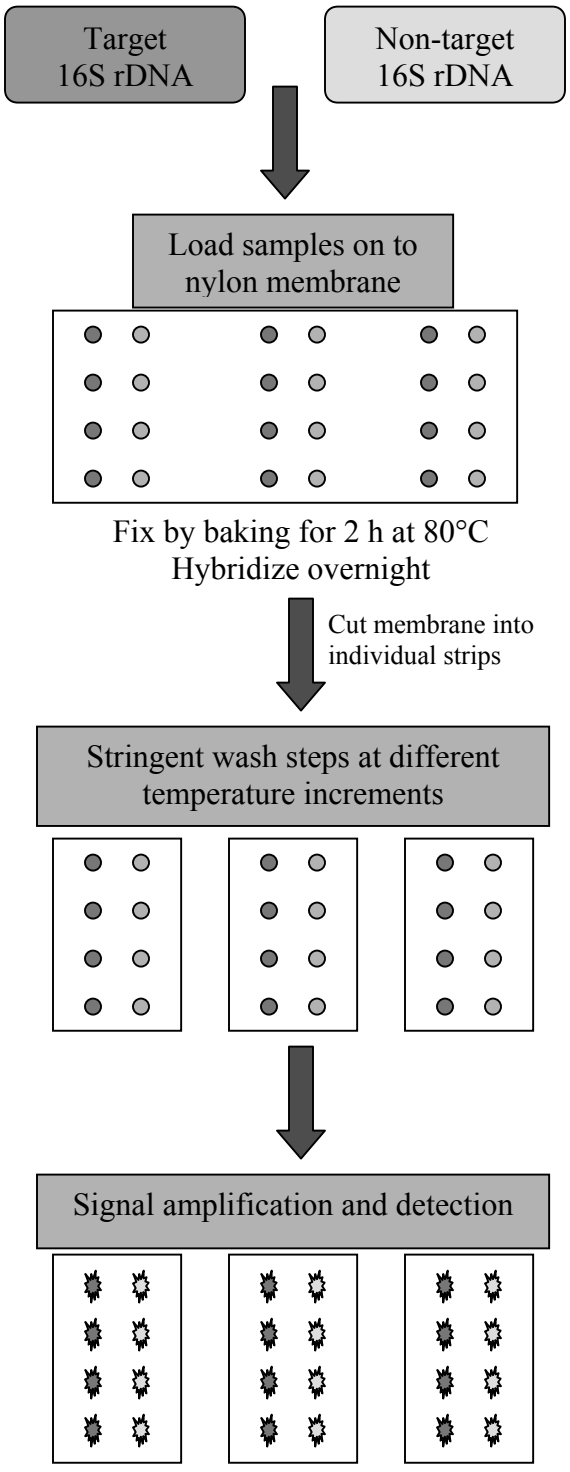


Figure 5. **Cartoon model of the probe optimization protocol**.

CHAPTER THREE

RESULTS AND DISCUSSION

PCR-amplification of 16S rDNA from a mixed population

Multiple complex bacterial cultures were analyzed throughout this study, including the nitrifying bioaugmentation culture 1010N, activated sludge from a laboratory-scale bioreactor, and activated sludge from a wastewater treatment plant. Primers 341f-GC and 534r were used to PCR-amplify the variable V3 region (Muyzer *et al*., 1993) of the 16S rRNA genes of the bacterial strains present in these cultures for subsequent TGGE analysis. One commonly encountered difficulty when using conserved primers to amplify small sections of the 16S rDNA within a mixed pool is the formation of erroneous PCR products. As shown in Figure 6, the "touchdown PCR" method (Don *et al*., 1991) effectively eliminated nonspecific annealing of primers to non-target DNA when amplifying 1010N. This PCR method was used to amplify the 200 bp region of interest from genomic DNA of all complex populations analyzed via TGGE in this study.

In addition to the optimal temperature conditions of the PCR reaction, the optimal number of cycles was also determined. A series of PCR reactions were set up with identical template (3 ng of pNS15 and 3 ng of pMP15), in which the number of cycles were varied from 15, 17, 20, 25, 30, 35, and 50. It was observed that at 30 cycles, the reaction was within the linear range and the ratio between the two products was equivalent to the starting ratio (data not shown). After 35 cycles the reaction began to enter plateau-phase, and the ratio of pNS15 to pMP15 amplification products was

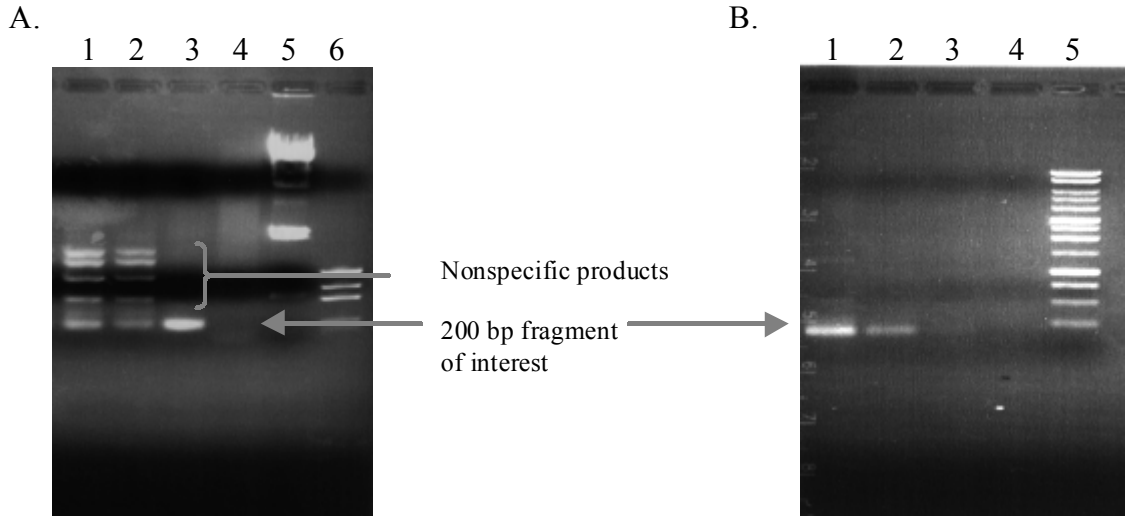
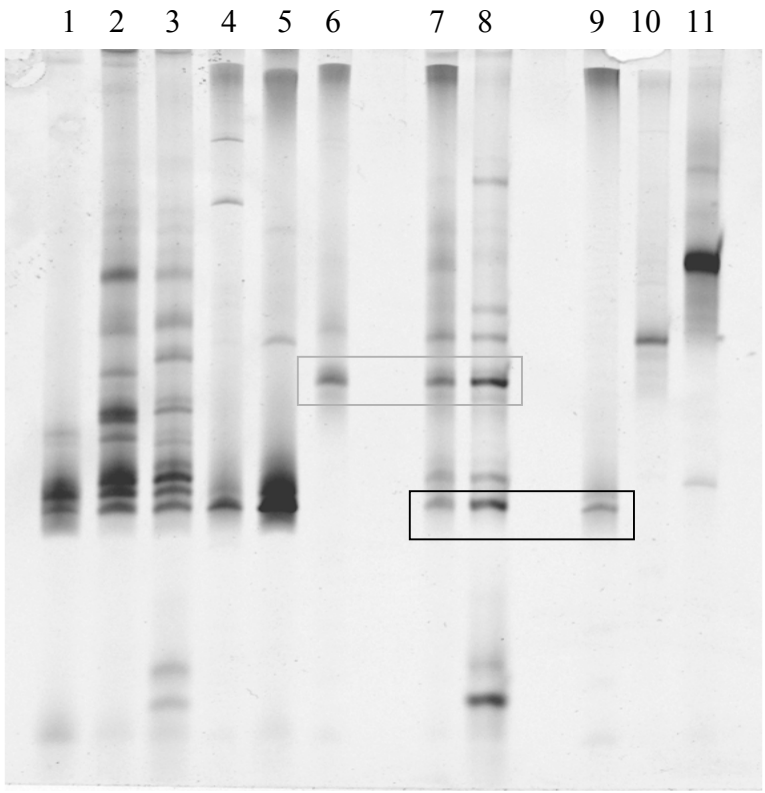


Figure 6. Touchdown PCR. Panel A illustrates products generated from a standard PCR amplification of serial dilutions of genomic DNA extractions from the mixed bacterial consortium, 1010N, in lanes 1 (genomic DNA template diluted 1:10) and 2 (1:100). Lane 3 is a pure culture isolate, *E. coli* DH5α, used for a positive control. Lane 4 is the negative control, lane 5 is a λ-HindIII standard, and lane 6 is a molecular mass standard (Bio-Rad). Panel B illustrates products from a PCR amplification of serial dilutions of genomic 1010N DNA using the "touchdown PCR" method in lanes 1 (1:10), 2 (1:100), and 3 (1:1000). Lane 4 is the negative control and lane 5 is a 1 Kb DNA ladder (Promega). Both PCR reactions were carried out using primers 341f-GC and 534r. From a 100 μl total volume PCR reaction, 10 μl were loaded in each lane.

altered (data not shown). A similar experiment was performed by Brüggeman, *et al*. (2000) in which the results were comparable. At 30 cycles the PCR reaction is not limiting, nor does it misrepresent the ratio of amplification products from different templates.

TGGE analysis of pure cultures and recently developed bioaugmentation cultures

Various cultures provided by Sybron were analyzed through PCR amplification and TGGE analysis, including ATCC strains, strains isolated from commercially prepared mixed microbial cultures, and bioaugmentation cultures that have been more recently developed. These strains were a variety of different ammonia- and nitrite-oxidizers. Comparison of the banding pattern of these pure cultures as they were visualized on a TGGE gel (Figure 7) indicates a group-specific behavior. A general trend in migration patterns of ammonia-oxidizing bacteria versus nitrite-oxidizing bacteria was observed where the melting domains of the latter have a slightly higher melting temperature, and therefore migrate further into the gel. Two of Sybron's more recently developed bioaugmentation cultures, B2 (6:2) and B2 (7:2), were designed to be more efficient in the oxidation of nitrite, which is the intermediate in the two-step nitrification process that has toxic properties when allowed to accumulate. The banding pattern of these two cultures indicates at least nine predominant isolates, with a more diverse population of NOB than 1010N based on the migration of the bands. This implicates the potential for increased levels of nitrite oxidation when added to a system; however, further analysis of the activity of these cultures needs to be completed. It is also important to note that the banding pattern visualized with TGGE analysis, when studying



1. KH3 - NOB
2. B2 (6:2) – bioaugmentation culture
3. B2 (7:2) – bioaugmentation culture
4. *Nitrobacter sp*. ATCC 25384
5. *Nitrobacter winogradskyi* ATCC 25391
6. NS500-9 - AOB from 1010N
7. 1010N LOT # 8462132A
8. 1010N LOT # 936L942
9. MPN2 - NOB from 1010N
10. 244-7-3 - AOB
11. *Nitrosomonas europaea* ATCC 19718

Figure 7. TGGE analysis of Sybron's bioaugmentation cultures. Lanes 1, 4, 5, and 9 illustrate PCR products generated from pure culture isolates of nitrite oxidizing bacteria. Lane 1; strain KH3 was isolated from a mixed culture at Sybron. Lane 4; *Nitrobacter* ATCC 25384. Lane 5; *Nitrobacter winogradskyi* ATCC 25391. Lane 9; MPN2 was isolated from 1010N. Lanes 6, 10, and 11 are pure culture isolates of ammonia-oxidizing bacteria. Lane 6; NS500-9 was isolated from 1010N. Lane 10; strain 244-7-3 was isolated from another mixed culture at Sybron. Lane 11; *Nitrosomonas* ATCC 19718. Lanes 2 and 3 illustrate PCR products generated from bioaugmentation cultures B2(6:2) and B2(7:2), respectively, that were enriched for nitrifying bacteria by Sybron. Lanes 7 and 8 illustrate PCR products generated from two different batches of 1010N. The PCR product of pure culture isolate NS500-9 (lane 6) and the band from 1010N having the same mobility are indicated by the light gray rectangle. The PCR product of pure culture isolate MPN2 (lane 9) and the band from 1010N having the same mobility are indicated by the black rectangle. All samples were amplified using the "touchdown PCR" method. From a 100 µl total volume PCR reaction, 10 µl were loaded in each lane (roughly 500-750 ng).

an unknown bacterial consortium, cannot be viewed as an absolute representation of every bacterial strain present due to inherent bias introduced by DNA extraction and PCR-amplification. Bacterial strains that are present in relatively low numbers or that are selectively excluded during the DNA purification and PCR steps may not be visualized on a TGGE gel. However, TGGE is an extremely useful tool in establishing a general population profile that can be used as a starting point for further phylogenetic analysis of the predominant bacterial isolates.

The bacterial composition of 1010N

The bacterial composition of the nitrifying bioaugmentation culture, 1010N, was also characterized using PCR amplification followed by TGGE analysis (Figure 7). The banding pattern of the 1010N product indicates the presence of at least six or seven predominant isolates, as there is a slight variation between two different batch preparations of 1010N. However, two predominant bands present in these two batch preparations, as well as 2 additional batches analyzed (data not shown), were found to have the same mobility as bands from the two pure culture isolates, MPN2 and NS500-9. As previously mentioned, MPN2 and NS500-9 were isolated from the 1010N product. Therefore, this suggested that two of the predominant strains of 1010N (as visualized on the TGGE gel) were available in pure culture for more extensive phylogenetic analysis. Because there is a possibility that two fragments with different sequence could exhibit the same mobility on a TGGE gel, it is necessary to extract the bands of interest from the gel and compare their nucleotide sequence to ensure that each band contains only one species of amplification product. Extraction of these correlating bands from the TGGE gel,

followed by determination and alignment of their nucleotide sequences, confirmed that these pure culture isolates, NS500-9 and MPN2, were in fact the predominant isolates in 1010N.

Phylogenetic analysis of NS500-9 and MPN2

In order to allow for a more thorough phylogenetic analysis of NS500-9 and MPN2, the nearly full-length 16S rRNA genes of each organism were PCR-amplified and subsequently cloned into the pGEM-T vector system (Promega). The entire fragment of each 16S rRNA gene cloned was sequenced on both strands, along with another strand from a second isolate. For each organism, the SeqMan and MegAlign programs in Lasergene99 were used to assemble the 16S rRNA gene sequence (Figures 8 and 9). The BLAST program in NCBI was used to align the 16S rDNA sequence of NS500-9 with previously published sequence in the public database, including GenBank, EMBL, and DDBJ. These alignments show a 97% sequence identity (1469/1502 base pairs aligned) to *Nitrosomonas europaea* (gb AF037106), and a 94% identity (1264/1333) to *Nitrosococcus mobilis* (gb M96403), indicating a more distant relationship. It has been proposed that sequence similarity must be below 95% to qualify as evidence of a novel species (Amann *et al*., 1995), suggesting that NS500-9 may be another strain of *N. europaea.* Alignment of the 16S rDNA sequence of MPN2 with previously published sequence shows a close relationship to *Nitrobacter* spTH21 (gb AF080257), having a 99% sequence identity (1399/1405 base pairs aligned); and *Nitrobacter hamburgensis* (gb L1163.1), with a sequence identity of 98% (1423/1428 base pairs aligned). Further

AATTCACTAGTGATTAGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCATGCTTTACACA
TGCAAGTCGAACGGCAGCGGGGGCTTCGGCCTGCCGGCGAGTGGCGAACGGGTGAGTAATGC
ATCGGAACGTGTCCTTAAGTGGGGAATAACGCATCGAAAGATGTGCTAATACCGCATATCTCT
GAGGAGAAAAGCAGGGGATCGTAAGACCTTGCGCTAAAGGAGCGGCCGATGTCTGATTAGCT
AGCTGGTGGGGTAAAGGCTTACCAAGGCAACGATCAGTAGTTGGTCTGAGAGGACGGCCAAC
CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATTTTGGACA
ATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCT
CTTTTAGTCGGAAAGAAAGGATTGCAACGAATAATTGTGATTTATGACGGTACCGACAGAAA
AAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTAATCGGA
ATTACTGGGCGTAAAGGGTGCGCAGGCGGTTTTGCAAGTCAGATGTGAAAGCCCCGGGCTTA
ACCTGGGAATTGCGTTTGAAACTACAAAGCTAGAGTGCAGCAGAGGGGAGTGGAATTCCATG
TGTAGCAGTGAAATGCGTAGAGATGTGGAAGAACACCGATAGCGAAGGCAGCTCCCTGGGTT
GACACTGACGCTCATGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCTTGGTAGTCCA
CGCCCTAAACGATGTCAACTGGTTGTCGGATCTAATTAAGGATTTGGTAACGTAGCTAACGCG
TGAAGTTGCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCC
GCACAAGCGGTGGATTATGTGGATTAATTCGATGCAACGCGAAAAACCTTACCTACCCTTGAC
ATGCTTGGAATCTAATGGAGACATAAGAGTGCCCGAAAGGGAGCCAAGACACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGT
CACTAATTGCTATCATTTTTAATGAGCACTTTAGTGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCCTCATGGCCCTTATGGGTAGGGCTTCACACGTAATACAATGGCG
TGTACAGAGGGTTGCCAACCCGCGAGGGGGAGCCAATCTCAGAAAGCACGTCGTAGTCCGGA
TCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGCGGATCAGCATGTCGC
GGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGGGAGTGGTTTTCACCAG
AAGCAGGTAGCTTAACCGCAAGGAGGGCGCTTGCCACGGTGGGGGTCATGACTGGGGTGAAG
TCGTAACAAGGTAGCCGTA

Figure 8. NS500-9 16S rRNA gene sequence. Sequence is a total of 1516 base pairs, listed from 5'-3'. Fragment was PCR amplified using primers 27f and 1492, and cloned into the pGEM-T vector system (Promega) for sequence analysis.

AGGCGGCCGCGAATTCACTAGTGATTAGAGTTTGATCATGGCTCAGAGCGAACGCTGGCGGC
AGGCTTAACACATGCAAGTCGAACGGGCGTAGCAATACGTCAGTGGCAGACGGGTGAGTAAC
GCGTGGGAACGTACCTTTTGGTTCGGAACAACCCAGGGAAACTTGGGCTAATACCGGATAAG
CCCTTACGGGGAAAGATTTATCGCCGAAAGATCGGCCCGCGTCTGATTAGCTTGTTGGTGAGG
TAACGGCTCACCAAGGCGACGATCAGTAGCTGGTCTGAGAGGATGATCAGCCACATTGGGAC
TGAGACACGGCCCAAACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAG
CCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCCTAGGGTTGTAAAGCTCTTTTGTGCGGG
AAGATAATGACGGTACCGCAAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCCGCGGTAA
TACGAAGGGGGCTAGCGTTGCTCGGAATTACTGGGCGTAAAGGGTGCGTAGGCGGGTCTTTA
AGTCAGGGGTGAAATCCTGGAGCTCAACTCCAGAACTGCCTTTGATACTGAGGATCTTGAGTT
CGGGAGAGGTGAGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAACACC
AGTGGCGAAGGCGGCTCACTGGCCCGATACTGACGCTGAGGCACGAAAGCGTGGGGAGCAAA
CAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGCCCGTTAGTGGGTTTA
CTCACTAGTGGCGCAGCTAACGCTTTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAA
ACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGACGCAAC
GCGCAGAACCTTACCAGCCCTTGACATGTCCATGACCGGTCGCAGAGATGTGACCCTCTCTTC
GGAGCATGGAGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCCCGTCCTTAGTTGCTACCATTTAGTTGAGCACTCTAAGGAGAC
TGCCGGTGATAAGCCGCGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCT
GGGCTACACACGTGCTACAATGGCGGTGACAATGGGAAGCAAAGGGGTGACCCCTAGCAAAT
CTCAAAAAACCGTCTCAGTTCGGATTGGGCTCTGCAACCCGAGCCCATGAAGTTGGAATCGCT
AGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCA
CACCATGGGAGTTGGTTTTACCTGAAGGCGGTGCGCTAACCCGCAAGGGAGGCAGCCGACCA
CGGTAGGGTCAGCGACTGGGGTGAAGTCGTAACAAGGTAACCGTAAA

Figure 9. MPN2 16S rRNA gene sequence. Sequence is a total of 1483 base pairs, listed from 5'-3'. Fragment was PCR amplified using primers 27f and 1492, and cloned into the pGEM-T vector system (Promega) for sequence analysis.

analysis of NS500-9 and MPN2 will be necessary in order to determine their genus and species classification.

Design and optimization of probes specific to NS500-9 and MPN2

Using the information obtained from sequencing the cloned 16S rRNA genes of NS500-9 and MPN2 and alignment of these sequences with previously published sequences in the public databases, organism-specific probes were designed. A 27-base nucleic acid probe, NS5445, was designed to target NS500-9. A 17-base nucleic acid probe, MPN996, was designed to target MPN2 (for sequence of probes, see Table 1). The specificity of each probe was also checked with the Check_Probe program in RDP, in which no exact matching complements were found. For a comparison of target vs. the most closely related non-target sequences (as listed in the RDP-II database) for both probes, see Table 2. The two probes were ordered with a 5'-fluorescein tag for subsequent chemifluorescent detection.

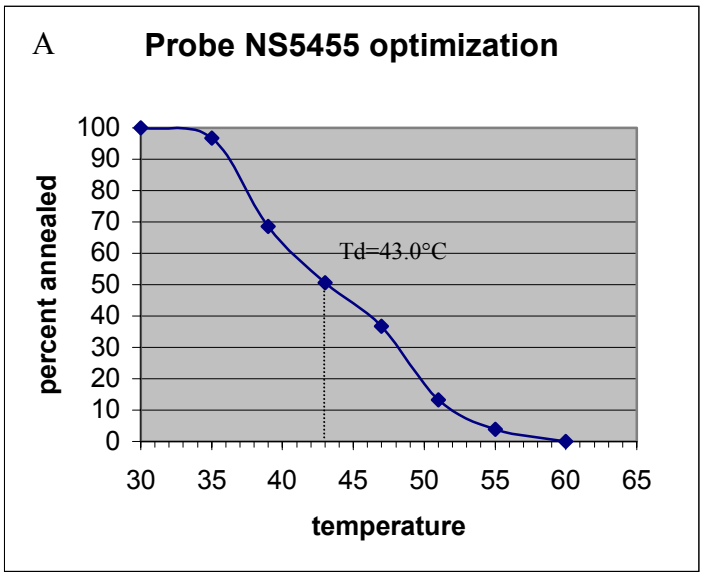
The temperature of dissociation (Td) is defined as the temperature at which one half of the bound probe is released from the probe-target hybrid. This value must be determined experimentally in order to find the optimum temperature at which to carry out the wash steps, given the stringency of the particular wash solutions. The Td is an essential factor in preventing the probe from binding to non-target nucleic acid. The results of the temperature optimization of probes NS5455 and MPN996 are shown in Figure 10. The experimental Td of NS5455 was determined to be 43°C, and the experimental Td of MPN996 was determined to be 40.5°C, as indicated by the melting

Table 2: Alignment of the 16S rDNA target regions of probes NS5445 and MPN996

Organism	Target of probe NS5445[a]
NS500-9	5’ -CGGAAAGAAAGGATTGCAACGAATAAT- 3’
Nitrosomonas europaea	5’ - • • • • • • • • • • • • AG • • • • • • T • • • • • • •-3’
Nitrosomonas europaea str. C-31	5’ - • • • • • • • • • • • • AG• • • • • • T • • • • • • • -3’
Nitrosococcus mobilis str. Nc2	5’ -T • • G • • • • • C • • • • • • • • • T • • • • • • • -3’

Organism	Target of probe MPN996[a]
MPN2	5’ -AGATGTGACCCTCTCTT- 3’
Nitrobacter winogradskyi	5’ - • • • • • • • • • • • T • • • • • • • - 3’
Bradyrhizobium sp. str. LMG 8295	5’ - • • • C • • • • • • T • • • • • • - 3’

a: • indicates the identical nucleotide as the above sequence



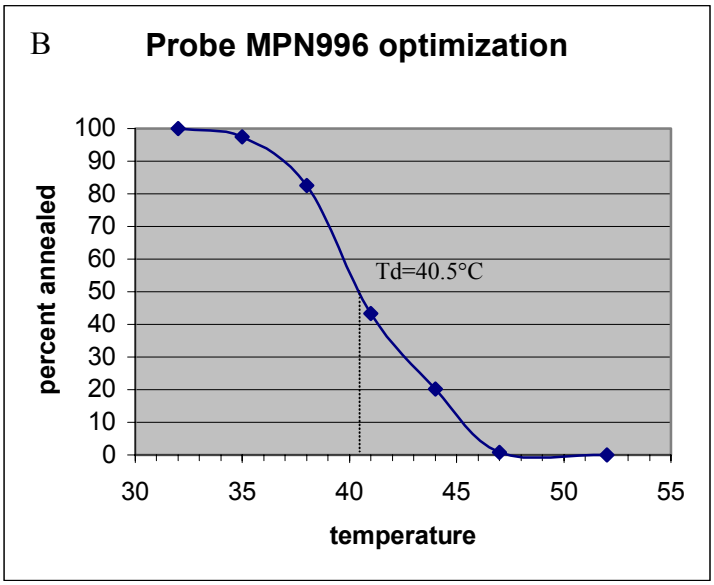


Figure 10. Temperature optimizations of probe NS5455 (A) and probe MPN996 (B). The experimental Td is designated by a dotted line. Plots are representation of experiments carried out in duplicate.

curve constructed from their annealing properties over the designated range of temperature.

The specificity of probe NS5455 was further tested by dot blot hybridization analysis. Duplicate membranes were prepared, each with target DNA that was PCR amplified (using primers 341f and 907r) from pNS15, and non-target DNA amplified from a genomic DNA extract of the closely related microorganism, *Nitrosomonas europaea* ATCC 19718. Although the exact sequence of the 16S rRNA gene is not known for this strain, it was assumed that it would be highly homologous to the sequence of NS500-9 in the region the probe would target, as are the two other strains of *Nitrosomonas europaea* whose sequences have been published. DNA samples were loaded in decreasing concentration, ranging from 1000 – 100 ng. Duplicate blots were hybridized separately with probes NS5455 (specific to NS500-9) and EUB338 (universal eubacterial probe) to test cross-reactivity of probe NS5455 (Figure 11). When the wash steps were carried out at the experimental Td (43.5°C), low levels of cross-reactivity were observed. By slightly elevating the temperature of the wash steps to 49°C, probe NS5455 showed no cross-reactivity with *Nitrosomonas europaea* DNA.

Isolation of rRNA

Quantitative dot blot hybridization is a technique whereby using carefully designed nucleic acid probes, may be used to determine the percentage of activity that can be attributed to one organism within a mixed population. This is done by calculating the ratio of 16S rRNA of that organism to the total 16S rRNA in the population, using specific and universal oligonucleotide probes. In this study, one objective was to

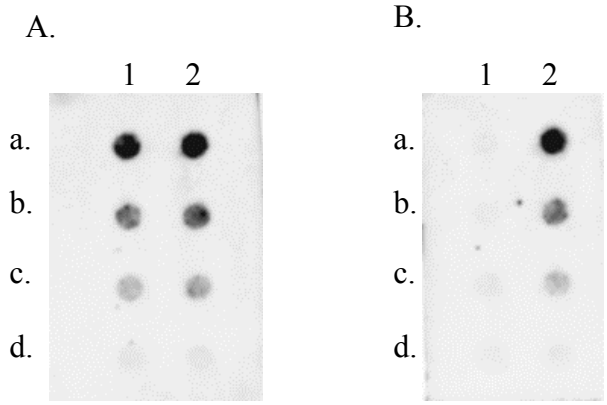


Figure 11. Dot blot hybridization analysis to test the specificity of probe NS455. PCR-amplified DNA (using primers 341f and 907r) from genomic DNA of *Nitrosomonas europaea* ATCC 19718 (lane 1) and from pNS15 (lane 2). 1000 ng, 500 ng, and 100 ng were loaded in rows a-c, respectively; while row d was the negative control (no DNA loaded). Membranes were hybridized overnight with the eubacterial probe EUB338 (A) and the NS5445 probe (B). Stringent wash steps were carried out at 49°C.

determine the percent activity of NS500-9 and MPN2 after the bioaugmentation culture was added to a system. Before analyzing the organisms within a complex environment, the rRNA purification protocols were optimized by performing extractions from actively growing pure cultures of MPN2, NS500-9, and *E.coli* DH5α. rRNA was successfully purified from each of these cultures (the results of an *E.co*li DH5α extraction are shown in Figure 12).

Once the rRNA purification technique was optimized, the next step was to attempt detection of the nucleic acid with the appropriate fluorescein-labeled probe. Immediately following the extraction, *E.coli* DH5α and NS500-9 rRNA were immobilized on the nylon membrane and subsequently hybridized with EUB338 and NS5455, respectively. However, in each experiment there was no detectable signal once the membrane was carried through the signal amplification protocol. In order to ensure the detection system was functioning, a positive control membrane loaded with 16S rDNA was included in every trial. In every trial, signal was detected on the positive control membrane The integrity of the rRNA was checked before and after loading the sample onto the membrane via electrophoresis in a 0.8% agarose gel (data not shown) in order to check for RNase contamination. In all cases the sample remained intact during the loading process. When the membrane was overloaded with rRNA (10 μg), a faint signal was detected (data not shown).

There are several potential explanations for the lack of signal when rRNA was the target. RNase contamination leading to sample degradation is always a concern when working with RNA. However, the fact that the integrity of the rRNA samples was checked by electrophoresis immediately before and after immobilization on the

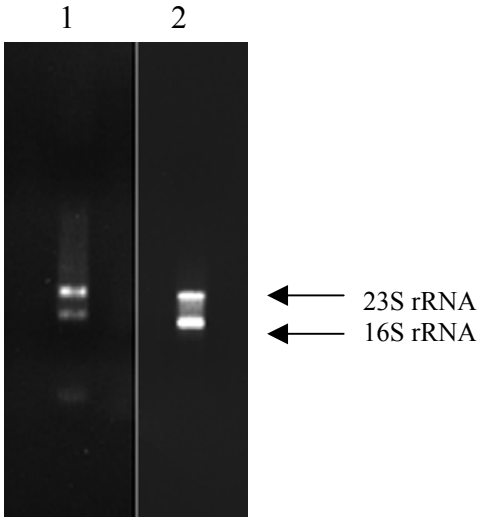


Figure 12. Purification of *E.coli* DH5α rRNA. Lane 1: approximately 2 μg rRNA extracted from an actively growing culture of *E.coli* DH5α. Lane 2: 4 μg of an *E.coli* rRNA standard (Boehringer Mannheim).

membrane and there was no visible degradation suggests that this was not the problem. Another possible problem could have been that the rRNA was being washed from the membrane, even after fixation by UV light or baking; but this also seems unlikely in light of the fact that the positive control DNA remained bound to the membrane. One other consideration was that the rRNA was not completely denatured, and secondary structure was blocking the probe from annealing efficiently. The application of alternate methods of denaturation, including heating and the addition of formamide, proved to be unsuccessful in enhancing signal detection. Related factors, such as secondary structure of the probe and probe-dimer formation, were eliminated by heating the probe before addition to the hybridization solution. After repeated efforts at troubleshooting, it was determined that efforts to quantify the percent activity attributable to NS500-9 and MPN2 within a system through rRNA analysis was not a feasible course of action for this study and efforts were directed towards a population study instead.

The use of an internal standard for template enumeration

Due to difficulties encountered with RNA-DNA hybridizations, an alternative method was investigated that would allow for a qualitative population analysis. This method involved the use of TGGE to calculate cell numbers in order to evaluate the stability of the newly introduced organisms after the bioaugmentation culture was added to a system. A study done by Brüggemann, *et al* (2000) demonstrates the use of an internal quantitative "standard" to be used in the analysis of environmental samples when employing PCR and TGGE/DGGE. The standard is based on the 16S rRNA gene of a naturally occurring organism (the bacterial X-endosymbiont of the mulberry psyllid

Anomoneura mori (Fukatsu and Nikoh, 1998)), which has an exceptionally high A-T content. Plasmid pCR2.1, containing the sequence encoding the standard (pCR2.1:std), was provided by Brüggeman *et al*. (2000) for use in this study. The appropriate fragment needed for use as a standard in TGGE was generated via PCR using the same primers, 341f-GC and 534r, that are used in this study. As a result of the A-T content, the standard has a relatively low Tm and migrates much higher in the gel than fragments amplified from other organisms, making it easy to visualize (see Figure 13).

In Brüggeman's study, a known number of molecules of the standard was added to a PCR reaction where a mixed environmental sample was being amplified and subsequently analyzed via DGGE. It was demonstrated that the original number of unknown template molecules could be calculated by comparing the intensity of the bands on the DGGE gel with that of the standard. In order to test the plausibility of using this standard as a means to quantify our system, competitive PCR reactions were set up in which the standard was amplified together with either pNS15, pMP15, or *E.coli* DH5α genomic DNA, and then one reaction that combined all four types of template. The TGGE analysis of these PCR reactions is shown in Figure 13. Preferential binding of primers 341f-GC and 534r to pNS15 and pMP15 over the standard was observed. Because the 3 plasmids were approximately the same size, copy number was not a determining factor. When amplified with *E.coli* DH5α, pNS15, and pMP15, the intensity of the standard band was significantly reduced to an almost undetectable level.

In the original study by Brüggeman *et al*, preferential binding was also observed, but to a lesser extent. The standard was repeatedly used to quantify the amount of PCR product (as visualized on the DGGE gel), that was amplified from a known number of

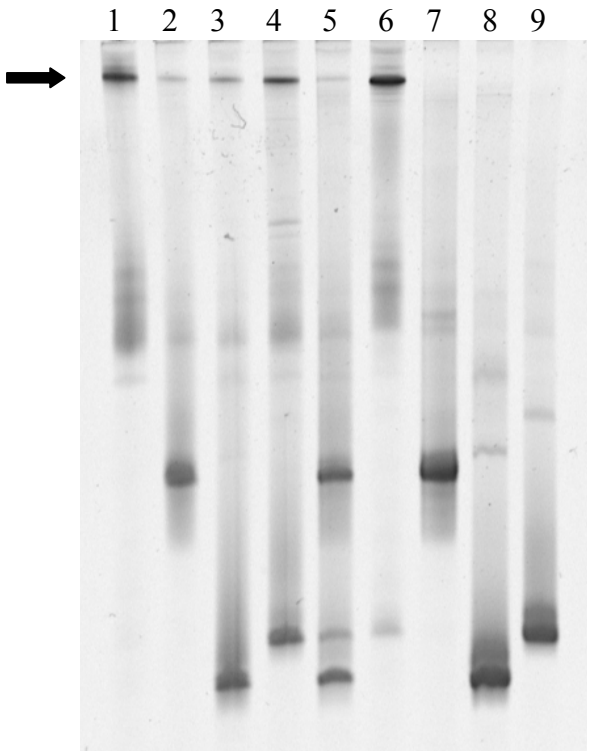


Figure 13. TGGE analysis of a competitive PCR reaction to test the internal standard. The template for the original PCR reactions were prepared as follows. Lane 1: 3 ng of plasmid pCR2.1 containing the sequence encoding the standard (pCR2.1:std) alone, indicated by the arrow. Lane 2: 3 ng of pCR2.1:std and 3 ng of pNS15. Lane 3: 3 ng of pCR2.1:std and 3 ng of pMP15. Lane 4: 3 ng of pCR2.1:std and 100 ng genomic *E.coli* DH5α DNA. Lane 5: 3ng of each plasmid template and 100 ng of genomic *E.coli* DH5α. The pCR2.1:std, pNS15, pMP15, and *E.coli* DH5α (lanes 6-9 respectively) were also amplified alone. The touchdown PCR was used with primers 341f-GC and 534r. In each lane, 10 μl of a 100 μl PCR reaction was applied to the gel. These results are representative of experiments done in duplicate.

D.vulgaris cells. The cell count calculated using the standard was compared to the actual cell count, which was consistently inaccurate. A correction factor of 2.5 was established to account for the preferential binding of the primers. Unfortunately, the binding preference of the primers in the present study was too severe to be adjusted with a correction factor, due to the fact that the standard was nearly undetectable when amplified in competition with the other templates used. A second complication in determining a correction factor for our system was the difficulty in obtaining accurate cell counts of nitrifying bacteria.

TGGE analysis of samples taken from a laboratory-scale sequencing batch reactor

Once it was concluded that the internal standard could not be used for quantitation in this study, an experiment was set up in which TGGE was used to determine whether a change in activity in a system could be correlated to a detectable increase in cell numbers of an organism. Mixed liquor from the Blacksburg Wastewater Treatment Facility (Blacksburg, VA) was used to set up two sequencing batch reactors (SBRs), in the laboratory of Dr. Nancy Love; one was augmented with 1010N and the other served as a control. Bioaugmentation was carried out daily over a period of 6 days starting at day 0, on a total per volume basis of 1%. The experiment lasted approximately 32 days, during which time ammonia concentrations, nitrate concentrations, and nitrate production rates were monitored in both reactors. Higher rates of nitrification (nitrate production rates) were observed in the augmented reactor in comparison to the control reactor (Rhodes, 2000).

Samples taken at day –1 (before augmentation), day 3 (middle of augmentation), and day 13 (highest measured nitrate production rate) from the augmented reactor were further analyzed for their population profiles. A total DNA extraction was carried out, followed by PCR amplification using primers 341f-GC and 534r. These PCR products were then analyzed via TGGE (Figure 14) in order to determine if there was a detectable shift in population as a result of bioaugmentation. On days 3 and 13, it was observed that at increased rates of nitrate production, there was no detectable increase in cell numbers of NS500-9. At either day 3 or day 13, there was no visible band attributable to NS500-9, nor were there any other significant changes in banding pattern. In this experiment, 1010N was added at what was to be predicted to be about 1% of the total population (Rhodes, 2000), which is apparently below the limits of detection of the TGGE system. These results suggest that changes in metabolically significant activity are not always accurately reflected by TGGE analysis. In general, while one would suspect that an increase in activity would be attributable to an increase in cell numbers, our finding suggests that an increase in activity may not be directly proportional to an increase in cell number. However, final conclusions may be made only when the limits of detection are established, and it is certain that the number of cells being studied are within these limits.

Sensitivity of the TGGE system

In order to determine the limits of detection of the TGGE system, a sample of pNS15 DNA was PCR-amplified (with 341f-GC and 534r). The PCR product was purified and quantified using the spectrophotometer. The PCR-amplified DNA was

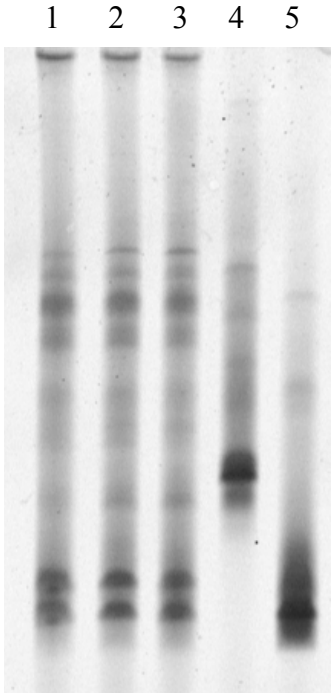


Figure 14. TGGE analysis of samples taken from a laboratory-scale bioaugmentation experiment. Lanes 1,2 and 3 illustrate the PCR products amplified from 100 ng of purified genomic DNA of samples taken at day –1, 3, and 13, respectively. Lanes 4 and 5 illustrate the PCR products amplified from 3 ng pNS15 and pMP15, respectively. All PCR products were generated using touchdown PCR with primers 341f-GC and 534r. In each lane, 10 µl of a 100 µl-PCR reaction (roughly 500-750 ng) was loaded into the gel. These results are representative of experiments done in duplicate.

applied to the gel in mass range of 10 ng – 1000 ng. It was observed that at least 10 ng of DNA (equivalent to 4.8 x 10^{10} molecules of PCR product) must be present in order to generate a detectable signal (as seen in Figure 15) when the gel is stained with Vistra Green (Amersham Pharmacia Biotech) and scanned with the Storm® 860 PhosphorImager (Molecular Dynamics) at 800 V.

In addition to the basic limits of detection, the sensitivity of the TGGE system was also examined for the ability to detect the bioaugmentation culture, 1010N, when added to activated sludge. A mixed liquor sample was taken from the Blacksburg Wastewater Treatment Facility (Blacksburg, VA) and used to set up a series of whole cell ratios of 1010N to activated sludge. The activated sludge was spiked with the 1010N culture on a per volume basis in the following percentages: 0.1%, 0.5%, 1%, 5%, 7.5%, 10%, 20%, and 50%. A total genomic DNA extraction was carried out for each of these combinations of 1010N and activated sludge. Primers 341f-GC and 534r were then used to PCR-amplify a region of the 16S rRNA gene for subsequent TGGE analysis (Figure 15). The band attributed to NS500-9 was used to detect the presence of 1010N because there was no band in the activated sludge that had the same mobility. It was observed that 1010N had to be present at a level of at least 5% of the total population (by volume) in order to detect the NS500-9 band. In addition, the percentage of NS500-9 in 1010N was analyzed by quantitating all of the 1010N bands on the TGGE gel (Figure 7) using the ImageQuant® software program. The intensity of the NS500-9 band was then compared to the collective intensity of all of the bands in 1010N, resulting in the estimation that NS500-9 band is 20% (or 1/5) of the total 1010N culture. Therefore, NS500-9 is detectable when it is present at approximately 1% of the total population (by volume).

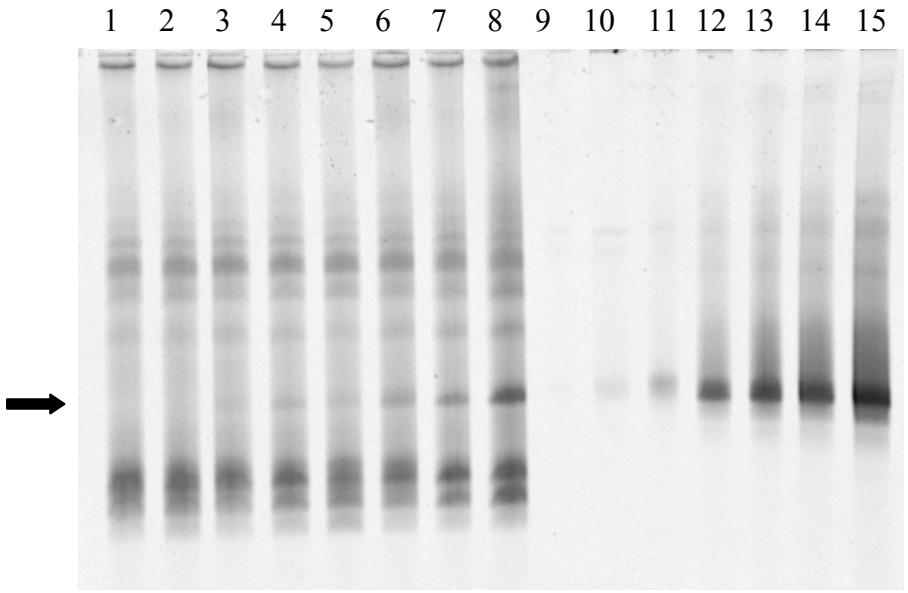


Figure 15. TGGE analysis of a whole cell spiking experiment. Lanes 1-8 illustrate PCR products amplified from genomic DNA purified from whole cells of activated sludge that had been spiked with whole cells of 1010N on a per volume basis in the following percentages, respectively: 0.5%, 1%, 5%, 7.5%, 10%, 15%, 20%, and 50%. In each lane, 10 µl of a 100 µl PCR reaction was applied to the gel. Lanes 9-15 illustrate PCR products amplified from pNS15 in the following range of mass, respectively: 10 ng, 50 ng, 100 ng, 250 ng, 500 ng, 750 ng, and 1000 ng. All PCR products were amplified with the touchdown PCR method, using primers 341f-GC and 534r. The arrow indicates the band attributable to NS500-9. These results are representative of experiments done in duplicate.

The efficiency of cell lysis and ensuing DNA purification is frequently named as a possible source of experimental bias in TGGE. In order to investigate this matter, the previous experiment was repeated with a slight alteration. Rather than combining whole cell samples on a per volume basis, purified genomic DNA of activated sludge was spiked with purified genomic DNA of 1010N on a per mass basis over a similar range of percentages (1%, 5%, 7.5%, 10%, 15%, 20%, and 50%). The purpose was to eliminate the efficiency of the extraction procedure as a determining factor. These mixed ratio templates were PCR-amplified (using primers 341f-GC and 534r) and subsequently analyzed via TGGE (Figure 16). The results matched those of the whole cell study, where the 1010N DNA had to be 5% of the total population of DNA in order for the NS500-9 band to be detectable. These results are evidence that volume and mass can be used interchangeably when comparing 1010N with the activated sludge samples used. This suggests that there were roughly equivalent cell counts/volume in the 1010N culture and the activated sludge samples as had been previously estimated theoretically (Rhodes, 2000). These results also demonstrate that cell lysis is not a significant source of bias in our system.

The PCR product amplified from pNS15 (using primers 341f-GC and 534r) in a range of mass (10 ng – 1000 ng), was used to construct a standard curve of mass vs. fluorescence for both the whole cell and genomic DNA spiking experiment (Table 3). In order to generate the PCR product for this standard curve, 3 ng of pNS15 (4.5 kb) was used for template DNA in duplicate PCR reactions. This starting mass was equivalent to 6×10^8 molecules of original plasmid molecules. After amplification using touchdown PCR, the two PCR reactions were combined, purified using the Qiagen PCR

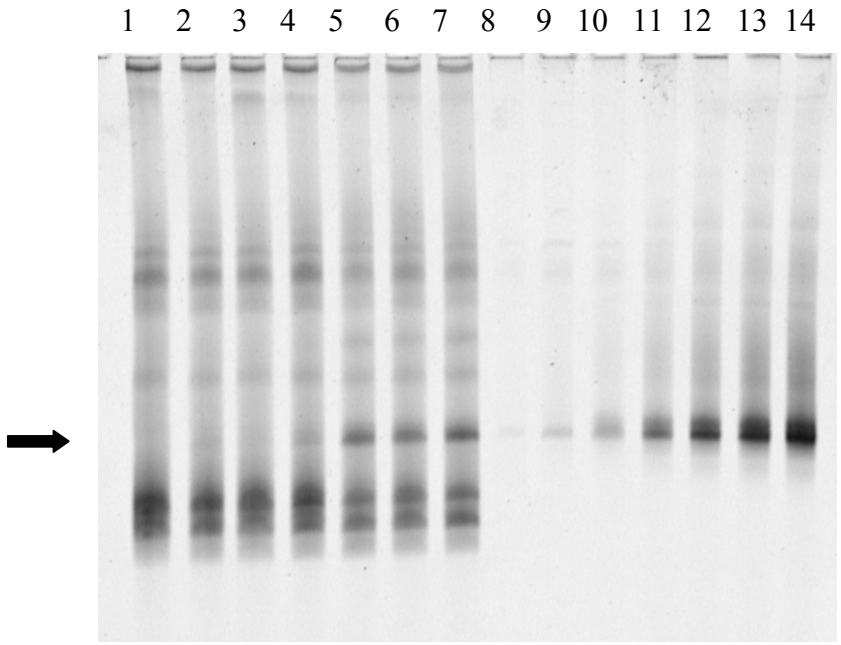


Figure 16. TGGE analysis of a genomic DNA spiking experiment. Lanes 1-7 illustrate PCR products amplified from genomic DNA of activated sludge that had been spiked with genomic DNA of 1010N on a per mass basis in the following percentages, repectively: 1%, 5%, 7.5%, 10%, 15%, 20%, and 50%. In each lane, 10 µl of a 100 µl PCR reaction were applied to the gel. Lanes 8-14 illustrate PCR products amplified from pNS15 in the following range of mass, respectively: 10 ng, 50 ng, 100 ng, 250 ng, 500 ng, 750 ng, and 1000 ng. All PCR products were amplified with the touchdown PCR method, using primers 341f-GC and 534r. The arrow indicates the band attributable to NS500-9. These results are representative of experiments done in duplicate.

Table 3: Standard curve relating mass to fluorescence using TGGE

Mass (ng)	Fluorescence (relative pixel density)
0	0
10	3720
50	25894
100	82088
250	271019
500	529152
750	725426
1000	898070

Purification Kit (Qiagen), and concentrated to a final volume of 50 μl. The concentration of this sample was measured at 100 ng/ μl using a spectrophotometer. By calculation, the total number of 200 bp molecules present after amplification was 1.1×10^{13} molecules. In order to determine whether the original number of template molecules could be estimated by calculating back from the final number of molecules, the equation $X_f = X_0 2^{30}$ was used to obtain the calculated original template number, 1×10^4 molecules. This number does not correlate to the actual number of original template molecules (6×10^8), which is likely due to the fact that by the very nature of touchdown PCR the amplification efficiency is well below 100% at each round of amplification.

The original goal was to use this standard curve to quantitate the NS500-9 band within the environmental sample in order to calculate the number of original template molecules, and thereby estimate cell numbers. Unfortunately, these calculations are evidence that this type of quantification was not possible. From this standard curve, however, it is possible to estimate the minimum number of cells that are necessary to detect signal with this system. On the TGGE gel, the lowest mass detectable was 10 ng, which was 0.004% of the total mass generated through the PCR reaction. On a theoretical basis, this indicates that 0.004% of the original template would be the minimum number of molecules necessary for detection, which is calculated to be 2.4×10^6 molecules. In order to express this number in terms of cells, the copy number of rRNA genes per cell must be considered. Because the rRNA gene copy number is not known for *Nitrosomonas sp.*, it can only be estimated that the number is between 1 and 14 (Cole and Girons, 1994). The result is a rough estimation that between 1.7×10^5 and

2.4×10^6 cells are the minimum number of cells required for detection using this system, assuming 100% efficiency of cell lysis and recovery of DNA.

Conclusions

The experiments performed throughout this study illustrate the advantages and disadvantages of using PCR and TGGE to study a complex population. Perhaps the greatest advantage is the ability to very quickly construct a basic profile of the predominant organisms that are present in an unknown system. In contrast to lengthy alternatives, such as random "shotgun" cloning where numerous steps must be taken in order to gather comprehensive information about the bacterial constituents, TGGE offers a rapid illustration of the dominant organisms. In addition, the information provided by TGGE may be utilized for the design of probes specific to a particular organism. These probes can then be used in a variety of applications, including whole colony hybridizations (in order to isolate an organism in pure culture), an activity analysis, or a more detailed *in situ* analysis. The efficiency with which so much progress can be made defines TGGE as an integral stepping stone in approaching an unknown system.

Along with the practical applications, the TGGE system does have some weaknesses. In analyzing an unknown system, it is probable that the 16S rDNA of organisms present in very low numbers will not be sufficiently PCR-amplified and, as a result, not be detected. Identification of every bacterial strain was not a goal in this study and, therefore, not a concern. There is also the possibility of PCR bias, which involves the differential amplification of rRNA genes within a mixed population. Experiments done in this study and other studies (Brüggeman *et al*., 2000) indicate that when the A+T

content of the organisms being analyzed is not substantially different, the resulting profile is representative of the dominant organisms. The most outstanding weakness of the TGGE system, as it was applied in this study, is the inability to correlate changes in activity with a shift in population. Due to limits in the sensitivity of the system, small metabolically significant changes within a population are undetectable. Furthermore, the activity of particular organisms within a system cannot be assessed by examining only changes in cell numbers. In order to evaluate activity it is necessary to analyze the rRNA content of the cells, which is directly correlated to the cellular activity. Therefore, the TGGE system is a useful tool in outlining a bacterial population, obtaining rough estimates of cell numbers, and designing nucleic acid probes that are specific to certain organisms. However, to determine the activity of an organism or group of organisms within a mixed population, the most effective approach is to utilize the designed probes in an *in situ* study (fluorescent *in situ* hybridization – FISH), to obtain information on both cell number and activity.

CHAPTER FOUR

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Graduate Teaching Assistant: Virginia Tech, Blacksburg, VA.

Spring 2000	General Microbiology Laboratory
Fall 2000	General Microbiology Laboratory
Spring 2001	General Microbiology Laboratory

HONORS AND AWARDS

1998 Phi Sigma Psi Honor Society
1998 Dean's List

PROFESSIONAL MEMBERSHIPS

American Association for the Advancement of Science

American Society for Microbiology

MEETING PRESENTATIONS

1999 American Society for Microbiology (VA branch) – oral presentation

1999 Microbiology Departmental Seminar

2000 American Society for Microbiology (General Meeting) – poster presentation

REFERENCES

Available upon request