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APPLICATION OF DNA MICROARRAYS TO MICROBIAL ECOLOGY RESEARCH: HISTORY, CHALLENGES, AND RECENT DEVELOPMENTS

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ABSTRACT

During the last three decades, molecular methods have dramatically expanded our view of microbial diversity in natural and engineered systems. A variety of molecular approaches, including both PCR-based and hybridization-based techniques, have been applied extensively to the analysis of complex microbial communities and have yielded new insights. However, the majority of molecular methods that are widely used in microbial ecology are limited in their ability to encompass the incredible diversity of microbial communities. DNA microarrays, which were first introduced in the early 1990s, are one of the fastest growing technologies in biology, and they offer tremendous potential for microbial ecologists. DNA microarrays consist of nucleic acids spotted within a very small area on some solid support, and they enable the immobilization and simultaneous hybridization of hundreds of thousands of nucleic acids. This represents a dramatically higher degree of multiplexing than is possible with other widely used technologies. In addition, microarrays offer the advantages of increased speed of detection, low cost, and the potential for automation. Microarray technology has been used extensively for measuring gene expression in a wide variety of organisms, including human cells, plants, yeast, and bacteria, but its application to microbial ecology has been more limited. There are significant challenges to the use of microarrays in microbial ecology studies, including optimization of specificity and sensitivity and quantification of targets. However, in recent years several research groups have made significant progress in overcoming these challenges, and microarrays are beginning to be applied more frequently to microbial ecology studies in a variety of systems including terrestrial soils, wetland sediments, and freshwater and marine ecosystems. This article will provide a brief history of the use of molecular methods in microbial ecology, and will then review the development of microarray technology, the challenges that exist for application of microarrays to microbial ecology, the available strategies for overcoming these challenges, and some recent applications of microarrays to studies in microbial ecology.

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Pure Culture Techniques

One of the key innovations that spurred the development of the science of microbiology was the pure culture technique, which was introduced by Robert Koch in the late 1800s (Beck, 2000). The pure culture technique made it possible for the first time to isolate individual microbial species in the lab and to study their physiological and biochemical properties, and it enabled Koch in 1876 to prove the germ theory of disease by demonstrating conclusively that *Bacillus anthracis* was the causative agent of anthrax (Koch, 1876). Subsequently Koch used the pure culture technique to identify other disease-causing microorganisms, including *Mycobacterium tuberculosis* and *Vibrio cholerae* (Beck, 2000), thus revolutionizing our understanding of human and animal disease.

The pure culture technique also allowed microbiologists to explore the incredible diversity of microorganisms in the natural world. In 1887 Sergei Winogradsky discovered the process of chemolithotrophy by isolating the marine bacterium *Beggiatoa* and observing that it could obtain the energy it needed for growth from the oxidation of an inorganic substrate (hydrogen sulfide), and in 1890 he elucidated another chemolithotrophic process when he isolated the first nitrifying bacteria from soil: *Nitrosomonas* and *Nitrosococcus* (Beck, 2000). In 1888 Martinus Beijerinck isolated the first nitrogen fixing bacterium (*Bacillus radicicola*) from the root nodules of the pea plant, and subsequently he isolated the first organism capable of anaerobic respiration: the sulfate reducing *Spirillum desulfuricans*, which is now known as *Desulfovibrio desulfuricans* (Beck, 2000).

More recently, Konneke et al. (2005) were the first to isolate an autotrophic ammonia-oxidizing Archaeon in pure culture. Autotrophic ammonia-oxidation was previously thought to be limited to the Bacterial domain, but the possibility of Archaeal ammonia oxidation was suggested by several recent studies: in a metagenomic analysis of the Sargasso Sea, Venter et al. (2004) found a unique ammonia monooxygenase gene (ammonia monooxygenase catalyzes the first step of ammonia oxidation) on an Archaeal-associated scaffold. In addition, Treusch et al. (2005) found genes encoding a potential ammonia monooxygenase on a metagenomic soil clone alongside an Archaeal ribosomal RNA operon. These studies suggested that an Archaeon might be able to oxidize ammonia, but it was not until Konneke et al. (2005) isolated the first pure culture of an Archaeal ammonia oxidizer, named *Nitrosopumilus maritimus*, that the ability of an Archaean to catalyze this process was conclusively proven.

Culture-based studies have thus made tremendous contributions to our understanding of microbial diversity and microbial physiology, and they continue to be a vital component of microbiology. However, culture-based studies, which require growth of microorganisms in the laboratory, are severely limited by the fact that most microorganisms are difficult or impossible to culture. Direct microscopic counts of bacteria in most habitats routinely exceed culture-based counts (plate counts or most probable number counts) by several orders of magnitude (Amann et al., 1995). For example, Jones (1977) found that the culturable fraction represented 0.25% of the total population in both freshwater and freshwater sediment, and Torsvik et al. (1990) reported that culturable organisms represented only 0.3% of the total community in soil. Staley and Konopka (1985) termed this phenomenon “the great plate count anomaly”, and this anomaly represents a significant challenge to microbial ecologists, as it indicates that culture-based studies provide an extremely restricted view of the diversity

of the natural microflora. Fortunately, molecular techniques, which do not rely on laboratory culture, have led to significant advances in the study of microbial communities (Kelly, 2003).

SSU rRNA TECHNIQUES

The most widely used molecular methods for the analysis of microbial communities are based on the small subunit ribosomal RNA (SSU rRNA) gene. The use of SSU rRNA as a phylogenetic marker was first proposed by Carl Woese in 1977 (Fox et al., 1977). The SSU rRNA molecule is a useful phylogenetic marker because it is present in all cells and because its sequence is fairly well conserved across phylogenetic groups. Since the pioneering work of Carl Woese, SSU rRNA gene sequences have been used to develop a comprehensive phylogenetic framework for the analysis of microbial communities (Amann et al., 1995). The most current version of the Ribosomal Database Project (release 9.56) contains 451,545 aligned and annotated SSU rRNA sequences (Cole et al., 2007).

Molecular techniques that analyze prokaryotic SSU rRNA (i.e. 16S rRNA) genes have been widely applied to the study of microbial ecology (Macrae, 2000), and there are a wide variety of methods that have been used. For example, a phylogenetic inventory of the prokaryotic component of a microbial community can be assembled using the Polymerase Chain Reaction (PCR) to amplify the 16S rRNA genes contained within the community, followed by cloning and sequencing this collection of amplified genes. This approach, known as clone library sequencing, was first applied by Giovannoni et al. (1990) for the analysis of marine microorganisms from the Sargasso Sea. Phylogenetic inventories of this type can be produced for the entire bacterial component of a microbial community by using "universal primers" designed to amplify 16S rRNA genes from all of the bacteria within a sample (e.g. Borneman et al., 1996; Kuske et al., 1997), or they can be produced for a specific phylogenetic group by using primers designed to amplify only the 16S rRNA genes of the group of interest. For example, Bruns et al. (1999) used primers specific for the 16S rRNA genes of autotrophic ammonia oxidizers to create an inventory of ammonia oxidizing bacteria from soil.

Clone library-based inventories have significantly increased our understanding of microbial diversity by revealing much greater diversity than was detected by classical culture-based studies (Becker et al., 2000). However, clone library preparation and sequencing is time consuming, labor intensive, and expensive. Although the cost and time required for large scale sequencing projects are decreasing quickly, the incredible diversity of microbial communities makes it impractical at this point to attempt to sequence every amplicon produced from PCR amplification of an environmental sample. For example, soil from a beech forest showed total bacterial counts of 1.5×10^{10} g soil^{-1} and approximately 4,000 unique bacterial genomes based on DNA reassociation analysis (Torsvik et al., 1990), and a more recent study used reassociation kinetics to determine that a pristine soil contained more than one million distinct genomes (Gans et al., 2005). Attempting to capture all of this biodiversity by sequencing a clone library is beyond our current capabilities. Therefore, although phylogenetic inventories of microbial communities based on cloning and sequencing 16S rRNA genes can provide a great deal of useful information, these inventories will generally be incomplete, so populations comprising very small fractions of the overall

community may not be detected. In addition, clone library sequencing is too slow to be useful for routine profiling and comparison of large numbers of environmental samples.

As an alternative to cloning and sequencing, techniques such as denaturing gradient gel electrophoresis (DGGE; Muyzer et al., 1993) and terminal restriction fragment length polymorphism analysis (T-RFLP; Liu et al, 1997) can be used to analyze the amplicons produced by PCR amplification of DNA extracted from a microbial community. DGGE and T-RFLP do not identify individual bacterial species. They instead generate profiles for microbial communities based on differences in the gene sequences of their constituents, and these profiles can be used to compare microbial communities and assess their degree of similarity or difference. DGGE and T-RFLP have been applied to 16S rRNA amplicons produced with universal PCR primers (e.g. Crump et al., 2003; Janus et al., 2005) and to amplicons produced with group-specific PCR primers (e.g. Cebron et al., 2004; Rosch and Bothe, 2005), and these approaches have been used to compare microbial communities from different habitats (e.g. Ludemann et al., 2000), and to assess changes in microbial communities over time (e.g. Duineveld et al., 1998), seasonal changes (e.g. Crump et al., 2003), and changes resulting from different experimental treatments (e.g. Klamer et al., 2002).

LIMITATIONS OF PCR-BASED METHODS

Clone libraries, DGGE, and T-RFLP are all useful techniques, but they are all reliant on PCR amplification, and there are significant limitations to all techniques that are based on PCR amplification of DNA extracted from environmental samples. First, PCR-based assays cannot provide reliable quantitative information on microbial community composition due to the potential for bias in PCR amplification. PCR bias can be caused by differences in DNA extraction efficiency (Miller et al., 1999), differences in gene copy number (Farrelly et al., 1995), and differences in the efficiency of the PCR reaction itself (Suzuki and Giovannoni, 1996). PCR bias can result in misrepresentation of phylogenetic diversity (Lueders and Friedrich, 2003), and thus it can be problematic in microbial ecology studies that rely on PCR. A second limitation of PCR-based assays is that DNA can persist in the environment outside of a cell for significant periods of time. For example, free DNA can bind to soil particles (Blum et al., 1997) and this binding can protect DNA from degradation (Demaneche et al., 2001). Thus, when working with environmental samples, it is possible for PCR to amplify DNA from cells that are no longer living, which would be a confounding factor in studies of microbial community structure. Both of these limitations, PCR bias and DNA persistence, should be considered when interpreting results from PCR-based community analyses such as clone library sequencing, T-RFLP, and DGGE (Kelly, 2003).

PROBE-BASED METHODS

As an alternative to PCR-based techniques, probe-based methods can be used to detect specific target bacteria in environmental samples. DNA probes, which are composed of short segments of DNA, typically 15 to 25 nucleotides in length, can be synthesized in the lab and

can be designed to target regions of the 16S rRNA gene that are unique to a particular phylogenetic group (Stahl, 1995). Ribosomal RNA is a good target for probing because active cells contain thousands to tens of thousands of copies of ribosomal RNA per cell, making it a naturally amplified target which can often be detected without PCR amplification (Amann and Ludwig, 2000).

DNA probes have generally been applied using either in-situ or membrane-based hybridization techniques. The in-situ technique, also known as fluorescence in-situ hybridization (FISH), hybridizes a fluorescently labeled probe to fixed whole cells. The fixation process renders the cells permeable to the probe, so cells containing RNA matched by the probe will be fluorescently labeled and can be observed and counted with an epifluorescence microscope. FISH, which was first developed by DeLong et al. (1989), can provide information on cell morphology as well as the abundance and spatial distribution of targeted organisms (Amann et al., 1995). FISH can be especially useful for examining spatial relationships between different phylogenetic groups of bacteria, as several unique probes can be applied to a sample simultaneously if the probes are labeled with different fluorescent markers (Mobarry et al., 1996).

DNA probes can also be utilized in a membrane hybridization format known as dot-blot hybridization, which is similar to a conventional Southern hybridization. In dot-blot hybridization, RNA isolated from a microbial community is immobilized on a nylon or nitrocellulose membrane filter and exposed to a radiolabeled probe. Hybridization of a probe to an immobilized target can be detected based on the radioactive tag and can provide information on the presence or absence as well as relative abundance of the group targeted by that probe. This approach was first applied to monitoring population changes in the rumen of cattle (Stahl et al., 1988), and since then it has been used extensively for the analysis of microbial communities from a variety of habitats (e.g. Raskin et al., 1996; Rooney-Varga et al., 1997; Weber, 2001). However, membrane hybridization has several significant limitations. Since the probes are labeled with radioactive tags, only one probe can be applied per membrane, so a separate membrane must be prepared for each probe, which can become quite cumbersome. In addition, membrane hybridization can provide information on relative rRNA abundance, but relative rRNA abundance can not be directly translated into cell numbers since cellular rRNA contents can vary significantly with growth rate (Amann et al., 1995).

In-situ and membrane hybridization techniques are extremely useful tools for microbial ecologists, and both methods have been widely applied. However, the scope of such studies is often constrained by the fact that these hybridization techniques severely limit the number of probes that can be applied simultaneously, thus limiting the amount of information that can be acquired (Kelly, 2003). Microarrays offer the opportunity to overcome some of the limitations of in-situ and membrane hybridization techniques.

DNA MICROARRAY TECHNOLOGY

DNA microarrays (also known as DNA microchips or DNA chips) generally consist of a set of nucleic acids spotted within a very small area on some solid support (Small et al., 2001). The concept of microarray hybridization of nucleic acids was first proposed 20 years

ago by Bains and Smith (1988) as a method for sequence determination. In 1994 Pease et al. built one of the first DNA microarrays using photolithography to synthesize a miniaturized array of 256 densely packed oligonucleotide probes on a glass surface (Pease et al., 1994). Pease et al. demonstrated that these surface immobilized oligonucleotides could be hybridized in parallel to fluorescently labeled oligonucleotide targets, and hybridization could be detected by epifluorescence microscopy. Pease et al. used their microarrays for sequencing by hybridization, as had been proposed by Bains and Smith (1988). In 1995 Schena et al. took a different approach and built a microarray by immobilizing 45 *Arabidosis thaliana* cDNAs on poly-L-lysine-coated microscope slides using a custom-built arraying machine with a single printing tip. All of the microarray-immobilized cDNAs were hybridized simultaneously with a mixture of fluorescently labeled cDNAs produced from total *Arabidopsis* mRNA by a single round of reverse transcription. Hybridization to the microarray-immobilized cDNAs was quantified by measuring fluorescence with a laser scanner. This microarray was able to quantify the relative expression of 45 *Arabidopsis* genes simultaneously (Schena et al., 1995). Since the pioneering work of Pease et al. (1994) and Schena et al. (1995), microarrays have been widely used: according to a search of Web of Science (Thompson Scientific, Philadelphia, PA) on February 1, 2008, Pease et al., (1994) had received 674 citations and Schena et al. (1995) had been cited 3,462 times. In addition, rapid developments in both robotics and miniaturization technology have made it possible to immobilize higher numbers of nucleic acids, from thousands to hundreds of thousands, in even smaller areas, thus providing extremely high hybridization capacity (Gentry et al., 2006).

The main application of microarrays to date has been analysis of gene expression (Gentry et al., 2006). In these studies, mRNAs are isolated from different samples or different experimental treatments and each mRNA pool is then either directly labeled with different fluorescent dyes (typically Cy3 and Cy5) (e.g. Taniguchi et al., 2001) or used to produce differentially labeled cDNAs via reverse transcription (e.g. Lashkari et al., 1997). Differentially labeled nucleic acid samples can then be hybridized simultaneously to a microarray containing gene specific probes, and comparison of the intensities of the two dyes provides relative expression levels of the genes contained on the microarray. This technique allows for the analysis of the differential expression of thousands of genes in a single experiment, and it has been used to analyze gene expression in a wide variety of organisms, including human cell lines (DeRisi et al., 1996), plants (Schena et al., 1995), yeast (Lashkari et al., 1997), and bacteria (de Saizieu et al., 1998). Microarrays have also been used for the detection of single nucleotide polymorphisms (Hacia, 1999; Straub, 2002), the detection of mutations (Cronin et al., 1996; Gerry et al., 1999), and the comparison of microbial genomes (Cho and Tiedje, 2001; Murray et al., 2001).

MICROARRAYS FOR MICROBIAL COMMUNITY ANALYSIS

David Stahl's group first proposed the use of microarrays for the detection of microbes within complex microbial communities (Guschin et al., 1997). The Guschin et al. approach was essentially the reverse of the dot-blot membrane hybridization technique in that Guschin et al. immobilized a set of oligonucleotide probes on a microarray, hybridized a fluorescently labeled target to all of these immobilized probes simultaneously, and detected hybridization

via epifluorescence microscopy. Guschin et al. successfully demonstrated this approach by fabricating a microarray that included a set of eight oligonucleotide probes targeting the 16S rRNA genes of several groups of nitrifying bacteria. When this microarray was hybridized with fluorescently labeled RNA from several reference strains, the immobilized probes were shown to selectively capture RNA from the appropriate target organisms, thus providing specific identification of the target organisms. Guschin et al. suggested that this approach could be used for the detection of different microbial populations within complex communities, and that the extremely high probe capacity of microarrays should allow for the detection of hundreds to thousands of target organisms simultaneously. This would represent much higher throughput than is possible with other probe hybridization formats (i.e. membrane hybridization and FISH).

After the pioneering work of Guschin et al. (1997), several groups demonstrated the effectiveness of microarrays for the detection of bacteria in complex communities based on hybridization of microarrays with RNA isolated directly from environmental samples: Small et al. (2001) detected *Geobacter* in soil samples; Koizumi et al. (2002) detected bacteria in oil-contaminated marine sediments; El Fantroussi et al. (2003) detected *Acidobacteria* as well as *Alpha*, *Beta*, and *Gamma Proteobacteria* in estuarine sediments; Kelly et al. (2005) detected nitrifying bacteria in wastewater treatment plant samples; and Smoot et al. (2005) detected bacteria in human saliva.

One significant advantage of microarrays for the analysis of complex microbial communities via 16S rRNA sequences is that their extremely high probe capacity enables researchers to create highly redundant and hierarchically nested probe sets. Redundant probes are multiple distinct probes that target the same organism or group; for example, in Figure 1 probes 1 and 2 are redundant probes for organism A and probes 3 and 4 are redundant probes for organism B. Hierarchically nested probes target organisms at multiple phylogenetic levels; for example, in Figure 1 probes 1, 6, and 7 are hierarchically nested. Due to the fact that the 16S rRNAs include both highly conserved and highly variable regions, it is possible to design hierarchically nested probes that target organisms at various phylogenetic levels (e.g. species, genus, phylum and domain), with highly conserved sequences giving broad taxonomic resolution and hypervariable sequences giving genus- and species-level resolution. For example, SSU rRNA probes have been designed for the highest taxonomic level, the domains Archaea, Bacteria, and Eukarya; for intermediate levels such as the alpha, beta, and gamma subclasses of *Proteobacteria*; and for many genera, species, and subspecies (Amann et al., 1995).

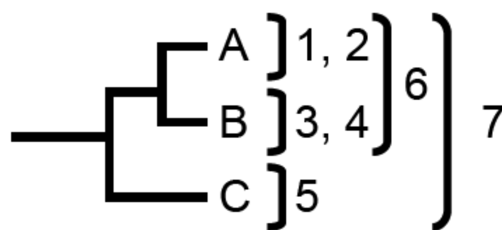

Figure 1. Model of a redundant and nested probe set. The model shows the phylogenetic realtionships of three hypothetical bacterial species (A, B, and C) as well as the coverage of hypothetical probes 1-7.

The use of redundant and hierarchically nested probes is not unique to microarray technology. These approaches are often incorporated to some degree in FISH and membrane hybridization experiments. However, the high probe capacity of microarrays enables redundant and hierarchically nested probes to be used on an unprecedented scale. Microarrays offer the possibility of including redundant and hierarchically nested probes for virtually every target, which would enable the detection of target organisms to be confirmed by multiple probes, thus greatly reducing false-positive errors. In addition, hierarchical nesting can provide a more complete picture of the overall phylogenetic composition of a microbial community than is possible if only genus- or species-specific probes are utilized, since broadly targeted probes will include organisms not targeted by more specific probes. A number of microarray studies have included both redundant and hierarchically nested probes (Liu et al., 2001; Loy et al., 2002; Kelly et al., 2002; Loy et al., 2005; Sanguin, Herrera et al., 2006).

MICROARRAY FORMATS

One interesting aspect of microarray technology is the fact that multiple formats are available. The most widely-used format is the planar or 2-D array (Starke et al., 2006) in which nucleic acids are immobilized on a glass slide in a single layer (e.g. Schena et al., 1995). Planar arrays are widely available because the equipment required for fabricating these arrays has been commercialized and is rapidly dropping in price due to competition between a number of manufacturers, making this format accessible to many laboratories (Li and Liu, 2003). An alternative to planar arrays are gel-based microarrays (also known as 3-D arrays) in which individual acrylamide gel pads or drops are arrayed on a glass slide, and nucleic acids are covalently cross-linked to the acrylamide within these three-dimensional gel pads (Yershov et al., 1996). Gel-pad microarrays have not been used as widely as planar arrays because these arrays are not available commercially and currently they are only fabricated by a limited number of research laboratories (Li and Liu, 2003). However, gel-pad microarrays do offer some advantages over planar arrays. For example, the three dimensional nature of the gel pads makes it possible to immobilize a much higher concentration of oligonucleotide probes in the same microarray surface area: planar arrays can immobilize a maximum of approximately 10 pmol/cm^2, whereas gel-pad microarrays can immobilize several nmols/cm^2 (Li and Liu, 2003). Higher probe concentration is significant as it can improve detection of low abundance targets (Starke et al., 2006).

One limitation of gel-pad microarrays is that they require targets to diffuse into the gel pad in order to hybridize with probes located within the gel interior, so the pore size of the gel will limit target access. Targets ranging in size from 20-150 nucleotides have been shown to penetrate the gel effectively and hybridize to probes throughout the entire three-dimensional gel pad (Starke et al., 2006). Therefore, when native RNAs or other large nucleic acids are used as the target for gel-pad microarrays, fragmentation of the nucleic acids is necessary prior to hybridization to allow access to the gel interior. Interestingly, fragmentation has also been shown to result in stronger hybridization signals for planar arrays (Liu et al., 2007). Fragmentation is beneficial in both gel-based and planar microarrays because it eliminates the three dimensional structure of the target nucleic acids which can interfere with hybridization,

and it reduces steric hindrance during hybridization which can occur with large target molecules (Liu et al., 2007).

Fragmentation of nucleic acids can be accomplished by treatment with divalent ions (Chee et al., 1996), alkali (Proudnikov and Mirzabekov, 1996), or nucleases (Gunderson, 1998). In addition, Kelly et al. (2002) developed an effective fragmentation method based on radical-generating coordination complexes. This method, which included simultaneous fluorescent labeling of the targets, resulted in random fragmentation of nucleic acids and effective hybridization of targets to DNA microarrays (Kelly et al., 2002). This method has been used in several microarray studies of environmental microbial communities (El Fantroussi et al., 2003; Smoot et al., 2005; Kelly et al., 2005; Siripong et al., 2006).

The gel used in manufacturing gel-pad microarrays can also be modified to increase its porosity. Several new gel polymers with significantly larger pore sizes are under development, which should help reduce the impact of the gel on target diffusion and should allow for the hybridization of larger targets (Starke et al, 2006).

FUNCTIONAL GENE MICROARRAYS

Bacteria can be placed into taxonomic groups based on phylogenetic affiliations (species, genera, etc.), but bacteria can also be grouped into functional guilds which can be defined by some metabolic capability (e.g. ability to denitrify) (Kelly, 2003). In some cases bacteria within a phylogenetically defined group share some metabolic functions. For example, among the bacteria autotrophic ammonia oxidation is restricted to two monophyletic groups: the first group belongs to the gamma subdivision of the Proteobacteria and includes one genus, *Nitrosococcus*; the second group belongs to the beta subdivision of the Proteobacteria and includes two genera: *Nitrosomonas* and *Nitrosospira* (Avrahami et al., 2002). In contrast, some metabolic functions are more widely distributed across numerous phylogenetic groups. An example is denitrification, which is found in about 50 bacterial genera (Rich et al., 2003). Molecular approaches based on 16S rRNA genes can target microorganisms based on their phylogenetic affiliation, which can provide functional information in some cases (e.g. ammonia oxidizing bacteria). However, 16S rRNA approaches are not useful for the detection of specific functional guilds when the function is widely distributed over the phylogenetic tree (e.g. denitrification) (Braker et al., 2001).

Functional guilds can be targeted using so called “functional genes” that code for enzymes critical to some metabolic process. Functional genes can be useful for detecting and monitoring functional guilds, and sequence variations within these functional genes can in some cases be used to differentiate members of a functional guild (e.g. Purkhold et al., 2000). Several prokaryotic functional genes involved in biogeochemical cycling processes have been identified and widely studied. For example, *amoA* codes for the membrane-associated active-site polypeptide of ammonia monooxygenase, which catalyzes the first step in nitrification (Rotthauwe et al. 1997); *nirS* and *nirK* code for structural variants of nitrite reductase, which catalyzes a key step in denitrification (Braker et al. 1998); *nifH* codes for one of the subunits of nitrogenase, the enzyme that catalyzes nitrogen fixation (Zehr et al., 2003) ; and *dsrA* and *dsrB* code for the alpha and beta subunits of dissimilatory sulfite reductase, which catalyzes a key step in sulfate reduction (Dahl et al., 1993). These functional genes have been used to

analyze microbial communities via many of the same molecular approaches discussed above, including clone library sequencing (Zehr et al., 1998; Braker et al. 2000), T-RFLP (Ohkuma et al ., 1999; Avrahami et al., 2002; Angeloni et al., 2006), and DGGE (Ibekwe et al., 2003).

A number of research groups have also applied microarray technology to the detection of functional genes. For example, Wu et al. (2001) built a functional gene microarray targeting different variants of several genes involved in the nitrogen cycle (*amoA*, *nirK,* and *nirS*). In this array, large PCR products (0.76 kb) from each of the functional gene variants were spotted on the array and used as hybridization probes. Taroncher-Oldenberg et al (2003) designed a microarray that included smaller oligonucleotide probes (70-mer) targeting different variants of *amoA*, *nifH, nirK,* and *nirS*. Zhou (2003) built a microarray that included 50-mer probes targeting genes involved in both nitrogen cycling (*nirS, nirK, nifH,* and *amoA*) and sulfur reduction (*dsrA* and *dsrB*), and Zhang et al. (2007) built an oligonucleotide microarray (15-25 mers) targeting variants of *nifH*.

CHALLENGES FOR MICROARRAY TECHNOLOGY: SPECIFICITY

The application of microarrays to the assessment of microbes in the environment poses a number of technical challenges. One of the main challenges is the specific detection of target nucleic acids against a complex background of non-target sequences. In any hybridization format, the potential exists for non-specific hybridization, i.e. hybridization between a target and probe that are not perfectly complementary. To achieve highly specific detection the discrimination of perfect and imperfect duplexes is important, and for detection of specific targets with short oligonucleotide probes this often requires the discrimination of single-base mismatches. In membrane-based hybridizations, single base mismatch discrimination can be achieved by optimizing the hybridization and wash conditions (buffer composition, salt concentration, and temperature) for each oligonucleotide probe (Stahl et al., 1988). This approach is effective for membrane hybridization since each membrane is hybridized with a single probe. However, the challenge for microarrays is that a large number of probes, which can vary in duplex stability due to differences in length and base composition, are hybridized and washed simultaneously under the same conditions, making it impossible to optimize the conditions for all of the probes on the microarray.

One approach to addressing this challenge is to design sets of probes such that all probes on an array have nearly identical melting temperatures. In this way, the microarray can be hybridized and washed under conditions (buffer composition, salt concentration, and temperature) that will minimize non-specific hybridizations for all of the probes. This approach can be effective (e.g. Bodrossy et al., 2003; Zhang et al., 2007), but it places significant constraints on probe design, which could make it difficult to build microarrays with very large numbers of probes.

Another approach to optimizing specificity is the inclusion of tetramethylammonium chloride (Maskos and Southern, 1993) or betaine (Rees et al. 1993) in the hybridization buffer. These compounds equalize the melting points of oligonucleotides with different base compositions by stabilizing AT base pairs (Peplies et al., 2003) so that a single wash temperature can be used for all probes on a microarray (for an example of this approach see Loy et al., 2002). This approach is reasonably effective and it is simple, but it does require

that all probes on the microarray be identical in length, which again places a constraint on probe design.

Specificity on microarrays can also be optimized via non-equilibrium dissociation kinetics, first demonstrated by Liu et al. (2001). In this approach, the microarray is hybridized with the targets, a thermal platform is used to control the temperature of the microarray, and the dissociation of targets from each of the probes is monitored simultaneously by measuring the fluorescent signal for each probe as the temperature of the array is incrementally increased. The resulting melting profiles (signal versus temperature) can be used to discriminate target hybridizations from non-target hybridizations, since duplexes containing one or more mismatches are generally less stable and dissociate at a lower temperature than perfect-match duplexes (e.g. see Figure 2). Liu et al (2001) first demonstrated the effectiveness of the non-equilibrium dissociation approach in gel-based microarrays, and Li et al. (2004) and Wick et al. (2006) subsequently confirmed its effectiveness in planar microarrays. The non-equilibrium dissociation approach has been applied to microarray analysis of microbial communities (Kelly et al., 2005; Siripong et al., 2006), so it has been shown to be effective, but this approach is time consuming and technically challenging. For example, effective discrimination of single base mismatches depends on mismatch position (Urakawa et al., 2003; Wick et al., 2006), which places constraints on probe design. In addition, the characterization and analysis of large numbers of melting profiles is difficult. However, several recent publications have developed software tools to address this challenge (Urakawa et al., 2002; Pozhitkov et al., 2005).

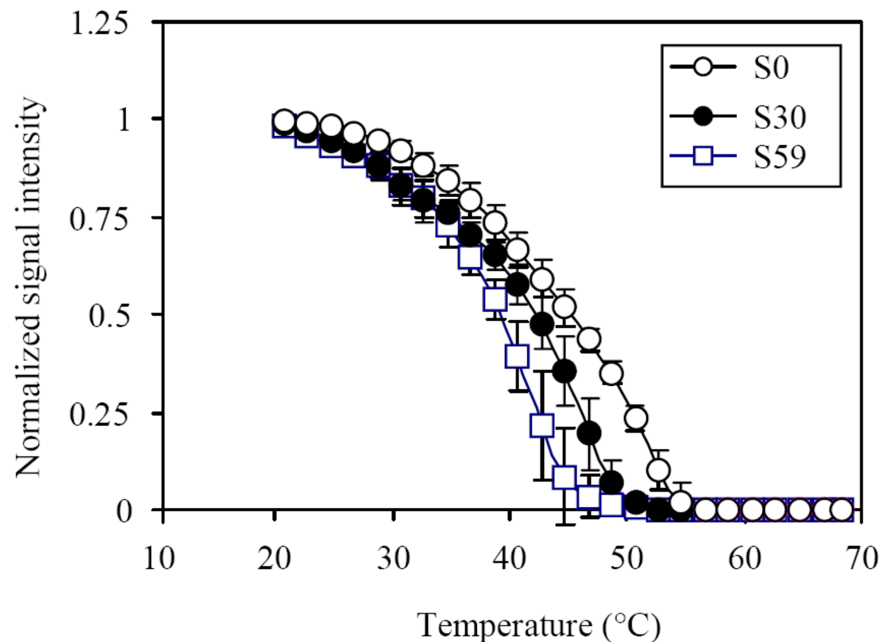

Figure 2.Example of non-equilibrium dissociation curves (signal versus temperature) obtained with a DNA microarray containing oligonucleotide probes. S0 represents a perfectly matched probe-target duplex, S30 represents a probe-target duplex with a single mismatch, and S59 represents a probe-target duplex with two mismatches. Data reprinted from Urakawa et al. (2003).

Another approach to optimizing specificity is the use of mismatch probes that are designed to differ in sequence relative to a perfect-match probe. Bavykin et al. (2008) used this strategy on a microarray that contained probes targeting 16S rRNA and 23S rRNA gene sequences in *Bacillus anthracis* and closely related microorganisms. This microarray was designed to include perfect match / mismatch probe pairs for each target, and Bavykin et al. found that analysis of perfect / mismatch signal ratios enabled specific identification of targets both singly and in mixtures (Bavykin et al., 2008). Wilson et al. (2002) used a similar approach with a SSU rRNA-based microarray and demonstrated specific detection of bacteria in pure cultures and simple mixtures.

Specificity can also be addressed by designing microarrays in which single base mismatch discrimination is not a necessity. For example, Zhou (2003) built a microarray which included 50-mer probes targeting specific variants of genes involved in nitrogen cycling (*nirS*, *nirK*, *nifH*, *amoA* and *pmoA*) and sulfur reduction (*dsrA* and *dsrB*). Zhou found that genes with less than 86–90% sequence identity were clearly differentiated using hybridization conditions of 50ºC and 50% formamide. Although this does not reflect a single base mismatch level of specificity, Zhou suggested that this level of specificity should provide species level resolution, since the average similarity of these functional genes at the species level ranged from 74 to 84% (Zhou 2003).

CHALLENGES FOR MICROARRAY TECHNOLOGY: SENSITIVITY

Sensitivity is another critical parameter for microarray applications. Planar microarrays have been shown to be 10^5-fold less sensitive than membrane hybridization (Cho and Tiedje, 2002), due to significant differences in the amount of nucleic acid immobilized: a membrane immobilizes > 1ug per dot, whereas a planar microarray immobilizes 10 to 20 pg per spot (Cho and Tiedje, 2002). As mentioned above, gel-pad microarrays have a higher probe binding capacity than planar arrays due to their three-dimensional nature. Gel-pad arrays have been used to immobilize 3 pmol of oligonucleotide probe per gel pad (Urakawa et al., 2002), which corresponds to approximately 18 ng for a typical 20 base pair probe. Therefore, gel-pad microarrays should result in an increase in sensitivity over planar arrays, but no direct comparisons of the sensitivity of these two formats are available in the literature.

Several design and methodological approaches can also be used to increase sensitivity, such as increasing probe length and reducing the stringency of the hybridization and/or wash conditions. Several studies have demonstrated that increasing probe length results in increased sensitivity of microarray hybridization (Zhou , 2003). In addition, a number of studies have demonstrated higher hybridization signals at lower stringency: Guschin et al. (1997) showed higher hybridization signals at lower hybridization temperatures, and several studies have reported higher hybridization signals at lower denaturant (formamide) concentrations (Urakawa et al., 2002; Zhang et al., 2007). However, increasing probe length and reducing stringency also have the drawback of decreasing hybridization specificity, so it is necessary in any microarray application to find an appropriate balance between specificity and sensitivity. Increasing probe concentration is another approach that has been shown to increase sensitivity (Guschin et al., 1997), and this approach has the advantage of not decreasing specificity.

Several groups have demonstrated that microarrays can be designed with adequate sensitivity to detect nucleic acids directly from environmental samples. As mentioned above, ribosomal RNA is a good target for hybridization because active cells contain thousands to tens of thousands of copies of ribosomal RNA per cell (Amann and Ludwig, 2000). Several groups have demonstrated successful microarray-based detection of ribosomal RNAs from a variety of environmental samples: Small et al. (2001) detected *Geobacter chapellei* 16S rRNA directly from a total-RNA soil extract; El Fantroussi et al. (2003) successfully detected *Acidobacteria* as well as *Alpha*, *Beta*, and *Gamma Proteobacteria* by hybridizing RNA extracted directly from estuarine sediments to a microarray containing 16S rRNA targeted oligonucleotide probes; and Kelly et al. (2005) detected nitrifying bacteria by hybridizing RNA extracted directly from a wastewater treatment plant aeration tank to a microarray containing 16S rRNA-targeted oligonucleotide probes.

Detection of bacterial DNA in environmental samples via direct hybridization is more challenging since most bacterial cells contain only a single chromosome which generally includes one or perhaps a few copies of each gene. Nevertheless, Wu et al. (2001) successfully hybridized DNA isolated from soil and sediment samples to a microarray using large PCR products (0.76 kb) as hybridization probes. These very large probes enabled high sensitivity (1 ng of target DNA), but low specificity (genes had to be at least 15% to 20% different in sequence in order to be discriminated). Zhou (2003) used much smaller oligonucleotide probes (50 mer) and successfully hybridized DNA isolated from soil. These shorter probes resulted in improved specificity (genes that were at least 10% to 14% different in sequence were discriminated), but lower sensitivity (8ng of target DNA). However, Zhou (2003) suggested that this level of sensitivity should be sufficient for many studies in microbial ecology since DNA yields from soil and sediment samples typically range between 10 and 400 μg of DNA per gram of soil dry weight.

AMPLIFICATION OF TARGETS PRIOR TO MICROARRAY HYBRIDIZATION

Although the studies cited above have shown some success in detecting bacteria in environmental samples by direct hybridization of either RNA or DNA, the sensitivity of these approaches may not be adequate to detect nucleic acids in low biomass systems or to detect nucleic acids that represent a small fraction of the total nucleic acid pool in a sample. For example, microarrays may not be sensitive enough to directly detect organisms whose rRNA makes up a small fraction of the total community rRNA pool either because the organisms are present in low numbers or because their cellular rRNA content is low due to a low level of metabolic activity. In addition, functional genes present in low concentrations may not be detectable by direct hybridization. For this reason, several groups have used PCR amplification prior to microarray hybridization to increase target concentration.

Because highly conserved universal primers for amplifying rRNA genes are available, PCR amplification can be run with universal 16S primers to simply increase the overall 16S rRNA gene concentration prior to hybridization with a microarray containing specific 16S rRNA probes. This approach was used by Loy et al. (2002) to detect sulfate-reducing prokaryotes in a hypersaline cyanobacterial mat, by Wang et al (2002) to detect a variety of

human intestinal bacteria in fecal samples, and by Loy et al. (2005) to detect members of the betaproteobacterial order "*Rhodocyclales"* in activated sludge from an industrial wastewater treatment plant. This approach has the advantage of increasing sensitivity, and general amplification of all 16S rRNA genes requires only a single PCR reaction.

PCR can also be run with group-specific primers to selectively amplify the 16S rRNA genes from specific phylogenetic groups. For example, Siripong et al. (2006) improved their detection of ammonia oxidizing bacteria (AOB) in wastewater treatment plant samples by selectively amplifying the 16S rRNA genes of beta-proteobacterial AOB with specific PCR primers prior to microarray hybridization, and Loy et al. (2005) found that the selective amplification of "*Rhodocyclales*" 16S rRNA genes prior to microarray hybridization allowed the detection of rare "*Rhodocyclales*" groups in activated sludge. Group-specific PCR amplification has the advantage of improving detection of the targeted group, but it does require separate PCR reactions for each group, which can limit the number of groups detected in a particular experiment.

Several groups have also used PCR amplification prior to microarray hybridization to detect functional genes in environmental samples. For example, Taroncher-Oldenberg et al. (2003) amplified *nirS* genes from sediment samples using general *nirS* primers and detected specific *nirS* variants by hybridization of amplicons to a microarray containing a set of 64 *nirS*-specific probes. Zhang et al. (2007) used a similar approach to detect *nifH* variants in roots of wild rice. One limitation of PCR amplification of functional genes prior to microarray hybridization is that each functional gene targeted by the array must be amplified via a separate PCR reaction. This was not a problem for the Zhang et al. (2007) study, as their array targeted only one functional gene, *nifH*, so universal *nifH* primers were used to amplify all *nifH* sequences in the samples followed by hybridization of amplicons to a set of 56 specific oligonucleotide probes on the microarray. However, the need for separate PCR reactions for each targeted gene could be problematic for microarrays targeting a large number of different functional genes. For example, the microarray used by Taroncher-Oldenberg et al (2003) included probes targeting four distinct functional genes (*amoA*, *nifH, nirK,* and *nirS*), each of which would have required a separate PCR reaction prior to microarray hybridization. Running multiple PCR reactions prior to microarray hybridization is not desirable for a number of reasons: 1) it will significantly increase the time and labor involved in microarray analysis, 2) it could introduce experimental error due to sample splitting, and 3) it limits the main advantage of DNA microarrays, namely the potential for highly parallel analysis of a high number of targets.

One way to avoid multiple PCR reactions is via Multiplex PCR, in which multiple primer sets are included in a single PCR reaction. Several groups have utilized multiplex PCR amplification prior to microarray hybridization. Panicker et al. (2004) demonstrated detection of *Vibrio parahaemolyticus* in shellfish using multiplex PCR amplification of four gene targets followed by microarray hybridization. Gonzalez et al. (2004) detected five marine fish pathogens by multiplexing nine primer sets and subsequently hybridizing PCR products to a microarray that included nine specific oligonucleotide probes. However, multiplex PCR is restricted in the number of targets that can be amplified simultaneously due to primer-primer interactions (Edwards and Gibbs, 1994). For example, Panicker et al. (2004) were able to multiplex four primer pairs targeting *Vibrio parahaemolyticus,* but were unable to multiplex ten primer pairs.

Pemov et al. (2005) developed a unique approach to multiplex PCR called multiplex microarray-enhanced PCR (MME-PCR) that avoids some of the issues associated with standard multiplex PCR. MME-PCR enables PCR amplification of multiple targets via specific primers immobilized within a gel-pad based microarray. MME-PCR prevents primer-primer interactions because each primer pair is immobilized within a separate gel-pad, so each primer pair is physically separated, and the specific amplification occurs within the gel pad. Amplification within each gel pad is detected by subsequent hybridization of fluorescently labeled, gene-specific probes. Pemov et al. (2005) demonstrated successful, specific on-chip amplification via MME-PCR of six genes from *Bacillus subtilis* using six different, gene-specific primer pairs. This approach could also be useful for many microbial ecology applications. For example, our lab is currently designing a MME-PCR chip for the detection of specific variants of nitrogen cycling functional genes.

Another way to avoid multiple PCR reactions prior to microarray hybridization is through a process known as multiple displacement amplification (MDA). The MDA reaction uses DNA polymerase and random primers to amplify entire genomes (Raghunathan, 2005). MDA has been used to amplify whole genomes from pure cell cultures (Raghunathan, 2005), and Wu et al. (2006) recently developed an MDA method for the amplification of entire genomes from mixed microbial communities, which they termed whole-community genome amplification (WCGA). Wu et al. (2006) used WCGA to amplify all of the genes in a microbial community prior to hybridization to an oligonucleotide (50 mer) functional gene array in order to improve sensitivity. They compared direct hybridization of genomic DNA isolated from groundwater to WCGA amplification of dilutions of this DNA prior to hybridization, and they found that WCGA-assisted microarray hybridization with as little as 1 ng of community DNA produced results that were highly similar to direct DNA hybridization. These results suggest that WCGA may be a useful method for improving the sensitivity of microarray-based assays for low biomass samples.

As demonstrated above, the addition of an amplification step to a microarray detection protocol can increase sensitivity and enable the detection of low abundance targets, but the incorporation of an amplification step does create some challenges as well. For example, double stranded nucleic acids such as PCR amplicons do not hybridize as effectively as single stranded nucleic acids (i.e. ssDNA or ssRNA) due to the potential for double stranded DNAs to reanneal instead of hybridizing to the microarray immobilized probes (Zhang et al., 2007). Several studies have avoided this problem by removing one of the DNA strands prior to hybridization via amplification with a 5'-biotin-labeled reverse primer and subsequent removal of this strand via streptavidin-coated paramagnetic beads (Peplies et al., 2003; Zhang et al., 2007). Another potential challenge related to amplification of targets prior to microarray hybridization is the fact that amplification may introduce biases, as was discussed above for PCR-based assays. Since an amplification step may not amplify all targets in an unbiased manner, amplification prior to microarray hybridization may interfere with the ability to extract quantitative information from microarray data.

CHALLENGES FOR MICROARRAY TECHNOLOGY: QUANTIFICATION

The value of microarrays as a tool for microbial ecologists would be greatly enhanced if microarrays could not only detect target nucleic acids but also provide quantitative information on their abundance. In theory, it should be possible to extract quantitative information from microarray hybridization if the amount of hybridization to a probe (i.e. the fluorescent intensity) correlates to the amount of target present in the sample. Several studies have confirmed linear relationships between target concentrations and signal intensities for specific probes: Wu et al (2001) found a log-linear relationship between the concentration of target genomic DNA and the hybridization signal for a 760 bp probe, and Rhee et al. (2004) found strong log-linear relationships between signal intensity and DNA concentrations for a large set of 50-mer oligonucleotide probes. Subsequently, Cho and Tiedje (2002) developed a method that involved co-immobilization of functional gene probes (500-900 bp) with a control probe (500 bp) and simultaneous hybridization of differentially labeled target genes and control DNA, and they achieved good log-linearity between signal ratio and DNA concentration ratio for three gene targets. These data confirmed that a correlation exists between the hybridization intensity for a microarray immobilized probe and the concentration of that probe’s target.

However, the challenge for microbial ecologists is that quantification of distinct bacterial populations in a complex community would require the comparison of hybridization signals from multiple probes with a microarray. This is a challenge because different probes can vary significantly in hybridization potential due to differences in probe length (Wu et al., 2001) and G+C content (Siripong et al., 2006) among other factors. These differences in hybridization potential have significant implications. Several studies have shown that redundant probes targeting the same organism often do not produce identical signal intensities when hybridized with RNA from the target organism (e.g. Peplies et al., 2003; Guschin et al., 2007). For example, Loy et al. (2005) demonstrated that probes targeting different regions of the 16S rRNA gene of the same organism can vary by a factor of 240 in signal intensity when hybridized with DNA from that organism. In addition, different probes can yield dramatically different hybridization signals even when hybridized to equal amounts of their respective targets (Ward et al., 2007). These situations make it extremely challenging to extract quantitative information on relative target abundance from multiple probes within a microarray. Since linear relationships have been shown to exist between target concentration and hybridization signal (Wu et al., 2001; Rhee et al., 2004), a standard curve could be developed for each probe on an array (Cho and Tiedje, 2002), as is routinely done for dot-blot membrane hybridizations. However, having to develop a standard curve for every probe on a microarray would be extremely labor intensive, and would limit the main advantage of microarrays, their high probe capacity. Therefore, attempting to determine the relative abundance of distinct bacterial populations in a complex community based on microarray hybridization is a very difficult task, and further work is needed to determine if microarrays can provide this type of information.

MICROARRAY-BASED MONITORING OF GENE EXPRESSION IN MIXED MICROBIAL COMMUNITIES

Functional gene arrays in which the presence of specific functional genes is detected via either direct hybridization of DNA extracted from an environmental sample (e.g. Zhou 2003) or via hybridization of functional genes amplified by PCR (e.g. Taroncher-Oldenberg et al., 2003; Zhang et al., 2007) can provide valuable information on the distribution of specific functional guilds in the environment as well as insight into the functional potential of microbial communities. However, the presence of a functional gene in an environmental sample does not necessarily mean that the gene is being expressed. One way to determine expression is by detection of gene transcripts (mRNAs). As discussed above, microarrays have been used extensively to investigate gene expression patterns in eukaryotic cells, such as human cell lines (DeRisi et al., 1996) and yeast (Lashkari et al., 1997), via analysis of mRNAs. Microarray analysis of mRNAs has been less extensively applied to prokaryotic cells, due to difficulties in priming cDNA synthesis from bacterial mRNA (Dennis et al., 2003). In addition, most microarray studies examining prokaryotic mRNA have been conducted under controlled laboratory conditions on single cell lines or pure bacterial cultures (e.g. de Saizieu et al., 1998; Methé et al., 2005) due to the difficulties associated with obtaining sufficient quantities of high quality mRNA from environmental samples (Parro et al., 2007).

One of the first studies to demonstrate the detection of bacterial mRNAs from mixed communities used a microarray containing near-full length amplicons from 25 catabolic genes involved in the degradation of chlorinated aromatic compounds (Dennis et al., 2003). The steps used in this study were extraction of total RNA, synthesis of cDNAs via reverse transcription, labeling of cDNAs, and hybridization of labeled cDNAs to the microarray. This approach demonstrated the induction of catabolic genes in response to substrate additions in pure cultures, in an artificial six member microbial community, and in sludge-fed pulp mill effluent (Dennis et al., 2003). Zhang et al. (2007) used a similar approach to detect expression of *nifH* gene variants in roots of wild rice samples with a microarray containing short (15-25 mer) oligonucleotide probes. Recently Parro et al. (2007) took a slightly different approach and built a DNA microarray containing a gene library from a single target organism, *Leptospirillum ferrooxidans*. They used this microarray to assess the relative expression of *L. ferrooxidans* genes in two habitats with low bacterial diversity that differed in salt and oxygen contents. The results demonstrated increased expression of genes required for adaptation to each of the two habitats: for example, genes related to halotolerance were preferentially expressed in the high salinity environment (Parro et al., 2007).

One of the challenges associated with microarray detection of bacterial mRNAs in environmental samples such as soil and sediments is the isolation of sufficient quantities of high quality mRNA for analysis (Parro et al., 2007). Several recent studies have addressed this challenge by developing strategies for amplification of low quantity bacterial mRNAs prior to microarray hybridization. Moreno-Paz and Parro (2006) used random-primed reverse transcription coupled to in vitro transcription as a method for total bacterial RNA amplification, and they demonstrated their ability to amplify as little as 250 ng of total bacterial RNA from a pure culture and successfully hybridize the product to a microarray. Gao et al. (2007) utilized a similar approach, which they termed whole-community RNA

amplification (WCRA), to amplify RNA from mixed bacterial communities. The WCRA approach used fusion primers (six to nine random nucleotides with an attached T7 promoter) for the first-strand synthesis, followed by second strand synthesis and in vitro transcription. This approach resulted in representative microarray detection from as little as 50 to 100 ng total RNA, and Gao et al. (2007) demonstrated that bacterial mRNAs could be detected in groundwater samples via WCRA amplification followed by hybridization to an oligonucleotide (50-mer) functional gene array.

The publications described above illustrate the potential for microarrays to monitor bacterial gene expression in environmental samples, which would provide extremely valuable insights into bacterial community function. The success of these pioneering studies should lead to further applications of this approach.

Parallel Analyses of Microbial Community Composition and Function with Microarrays

One of the key goals in microbial ecology research is the elucidation of the relationship between the composition of a microbial community (i.e. the species present) and the function of that community, but assessing this relationship for complex microbial communities in environmental samples has always been extremely challenging. As described above, numerous studies have demonstrated the ability of microarray technology to assess microbial community composition, via hybridization of either 16S rRNAs, 16S rRNA genes, or functional genes, and a few studies have demonstrated the ability of microarrays to assess microbial community function through the detection of mRNAs (Dennis et al., 2003; Gao et al., 2007; Parro et al., 2007). Several recent studies have expanded upon this work and developed highly innovative, microarray-based approaches to examine both microbial community composition and function in parallel.

Adamczyk et al. (2003) developed an isotope array approach. In this approach, a microbial community is incubated with a 14C-labelled substrate, and after an incubation period, total RNA is extracted from this community, fluorescently labeled, and hybridized with a microarray containing 16S targeted oligonucleotide probes. The array can then be scanned for fluorescence as well as for radioactivity. For each probe on the array, fluorescence indicates the presence of the target organism in the community, and radioactivity indicates that the target organism has incorporated 14C into RNA, indicating active growth and utilization of the labeled substrate. Adamczyk et al. (2003) demonstrated this approach using a microarray containing oligonucleotide probes targeting 16S rRNA genes of ammonia-oxidizing bacteria. In this study, activated-sludge samples were incubated with 14C-labelled bicarbonate, and detection of radioactivity for specific probes on the array was taken as an indication of CO_2 fixation by the targeted organisms. This study demonstrated the potential use of an isotope array for the simultaneous assessment of community composition and function.

Another novel approach to examining community composition and function was developed by Zhang et al. (2007). They assessed both the presence and expression of different variants of *nifH* in roots of wild rice using a microarray containing 56 oligonucleotide probes (15-25 mers) targeting *nifH* variants. In this study, DNA and RNA were coextracted from

plant roots, and separate aliquots were treated with either DNase I or RNase A to yield pure RNA and DNA samples, respectively. DNA was amplified prior to microarray hybridization via PCR with universal *nifH* primers. RT-PCR with a universal *nifH* primer was used to convert mRNA to cDNA, and PCR with universal *nifH* primers was then used to amplify the RT-PCR products prior to microarray hybridization. In this creative approach, hybridization of amplified DNA indicated the presence of specific *nifH* variants, and hybridization of amplified mRNA indicated the expression of specific *nifH* variants. The results of this study demonstrated that only a small subset of the *nifH*-containing organisms found in the roots were expressing the *nifH* gene (Zhang et al., 2007). This approach has tremendous potential for simultaneous exploration of microbial community composition and function, which could provide fascinating insights into the functioning of microbial communities in the environment.

APPLICATIONS OF DNA MICROARRAYS TO MICROBIAL ECOLOGY

Since the pioneering work of Guschin et al. (1997) the use of microarray technology in microbial ecology research has increased rapidly (Figure 3). The vast majority of microarray studies published to date in the field of microbial ecology have focused on demonstrating the capabilities of microarrays and developing the technology, but recently a growing number of studies have been applying microarrays as tools in studies asking ecological questions.

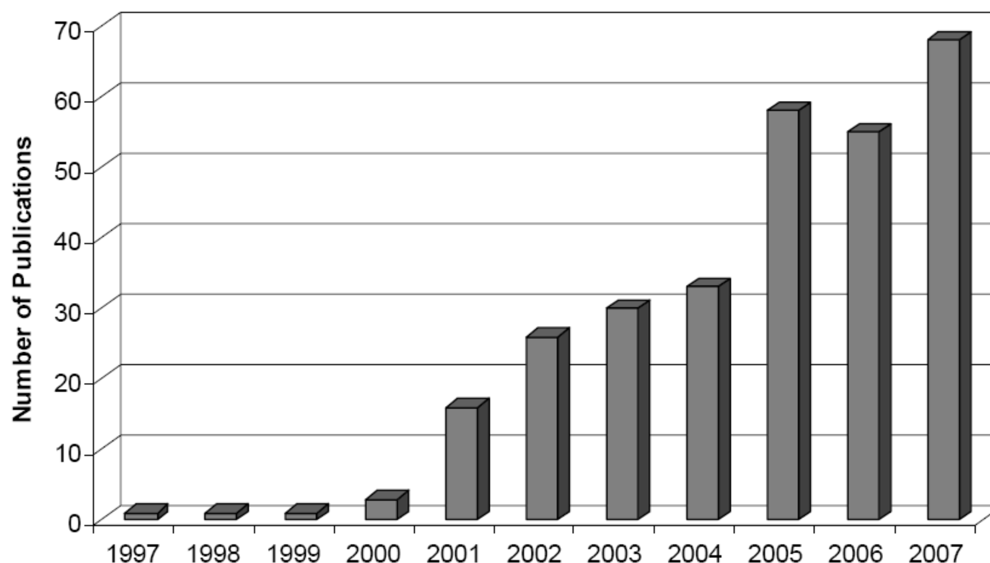

Figure 3.Number of publications per year that include application of microarray technology to microbial ecology. Numbers are based on a search of the Web of Science database on February 1, 2008 using the following search string: (TS=microarray* OR TS=microchip*) AND (TS=microbial ecology OR TS=microbial community OR TS=microbial detection OR TS=microbial identification). This approach was based on Wagner et al.(2007).

Some examples include the following: Taroncher-Oldenberg et al. (2003) used a microarray to reveal differences in the distribution of *nirS* variants in sediment samples from two stations in the Choptank River that differed in salinity, inorganic nitrogen, and dissolved organic carbon. Loy et al. (2002) used a 16S rRNA gene-based oligonucleotide microarray to detect sulfate-reducing prokaryotes in an unusual habitat, a low-sulfate acidic fen, and they found differences in the distribution of these sulfate-reducers in fen samples from different locations. Rhee et al. (2004) demonstrated that the community composition of naphthalene-degraders in soil microcosms differed depending on incubation conditions based on analysis with a functional-gene microarray containing oligonucleotide probes. Sanguin, Remenant, et al. (2006) used a 16S rRNA-based microarray to reveal a significant maize rhizosphere effect on soil bacterial community composition. Ward et al. (2007) demonstrated variations in the composition of ammonia oxidizing communities across a freshwater/marine transect extending from the Choptank River through the Chesapeake Bay and out into the Sargasso Sea using an oligonucleotide (70 mer) functional gene microarray targeting *amoA* genes. This study also revealed correlations between *amoA* guilds and environmental parameters, suggesting that different *amoA*-containing organisms occupy different ecological niches within the estuarine/marine environment (Ward et al., 2007).

All of the studies cited above are excellent illustrations of the effective use of microarrays in microbial ecology, and based on these and other successes it is likely that the uses of microarrays in this field will continue to expand rapidly.

Conclusions

Microarrays have the potential to revolutionize the field of microbial ecology via high throughput analysis of microbial community structure, function, and population dynamics. There are significant challenges to the use of microarrays in microbial ecology studies, including optimization of specificity and sensitivity and quantification of targets. However, as reviewed above, the last decade has seen tremendous progress in addressing these challenges, and this progress suggests that microbial ecologists are now on the verge of finally being able to realize the tremendous potential of microarray technology.

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References

Adamczyk, J, M Hesselsoe, N Iversen, M Horn, A Lehner, PH Nielsen, M Schloter, P Roslev, M Wagner (2003) The isotope array, a new tool that employs substrate-mediated labeling of rRNA for determination of microbial community structure and function. *Appl. Environ. Microbiol*. 69: 6875–6887.

Amann, RI (1995) Fluorescently labeled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Mol. Ecol.* 4:543–554.

Amann, RI, W Ludwig, K-H Schleifer (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev*. 59:143-169.

Amann, R, W Luwig (2000) Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology. *FEMS Microbiol. Rev.* 24: 555-565.

Angeloni, NL, KJ Jankowski, NC Tuchman, JJ Kelly (2006) Effects of an invasive cattail species (Typha x glauca) on sediment nitrogen and microbial community composition in a freshwater wetland. *FEMS Microbiol. Lett*. 263: 86-92.

Avrahami, S, R Conrad, G Braker (2002) Effect of soil ammonium concentration on N_2O release and on the community structure of ammonia oxidizers and denitrifiers. *Appl. Environ. Microbiol.* 68: 5685–5692.

Bains, W, GC Smith (1988) A novel method for nucleic acid sequence determination. *J. Theor. Biol.* 135: 303–308.

Bavykin, SG, VM Mikhailovich, VM Zakharyev, YP Lysov, JJ Kelly, OS Alferov, IM Gavin, AV Kukhtin, J Jackman, DA Stahl, D Chandler, AD Mirzabekov (2008) Discrimination of Bacillus anthracis and closely related microorganisms by analysis of 16S and 23S rRNA with oligonucleotide microarray. *Chem. Bio Interact*. 171: 212-235.

Beck, RW (2000) A chronology of microbiology in historical context. ASM press, Washington, D.C.

Becker S, P Boger, R Oehlmann, A Ernst (2000) PCR bias in ecological analysis: A case study for quantitative Taq nuclease assays in analyses of microbial communities. *Appl. Environ. Microbiol.* 66:4945-4953.

Blum, SAE, MG Lorenz, W Wackernagel (1997) Mechanism of retarded DNA degradation and prokaryotic origin of DNases in nonsterile soils. *Syst. Appl. Microbiol*. 20: 513-521.

Bodrossy L, N Stralis-Pavese, JC Murrell, S Radajewski, A Weilharter, A Sessitsch (2003) Development and validation of a diagnostic microbial microarray for methanotrophs. *Environ. Microbiol*. 5: 566–582.

Borneman, J, PW Skroch, KM O'Sullivan, JA Palus, NG Rumjanek, JL Jansen, J Nienhuis, EW Triplett (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* 62: 1935-1943.

Braker, G, A Fesefeldt, K-P Witzel1 (1998) Development of PCR primer systems for amplification of nitrite reductase genes (nirK and nirS) to detect denitrifying bacteria in environmental samples. *Appl. Environ. Microbiol*. 64: 3769-3775.

Braker, G, J Zhou, L Wu, AH Devol, JM Tiedje (2000) Nitrite reductase genes (nirK and nirS) as functional markers to investigate diversity of denitrifying bacteria in Pacific Northwest marine sediment communities. *Appl. Environ. Microbiol.* 66: 2096-2104.

Braker, G, H L Ayala-del-Rio, AH Devol, A Fesefeldt, JM Tiedje (2001) Community structure of denitrifiers, Bacteria, and Archaea along redox gradients in Pacific Northwest marine sediments by terminal restriction fragment length polymorphism analysis of amplified nitrite reductase (nirS) and 16S rRNA genes. *Appl. Environ. Microbiol.* 67:1893–1901.

Bruns, MA, JR Stephen, GA Kowalchuk, JI Prosser, EA Paul (1999) Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. *Appl. Environ. Microbiol*. 65: 2994-3000.

Cebron, A, M Coci, J Garnier, HJ Laanbroek (2004) Denaturing gradient gel electrophoretic analysis of ammonia-oxidizing bacterial community structure in the Lower Seine River: Impact of Paris wastewater effluents. *Appl. Environ. Microbiol*. 70: 6726–6737.

Chee, M, R Yang, E Hubbell, A Berno, XC Huang, D Stern, J Winkler, DJ Lockhart, MS Morris, SPA Fodor (1996) Accessing genetic information with high-density DNA arrays. *Scienc*e 274: 610–614.

Cho, JC, JM Tiedje (2001) Bacterial species determination from DNA-DNA hybridization using genome fragments and DNA microarrays. *Appl. Environ. Microbiol*. 67:3677–3682.

Cho, JC, JM Tiedje (2002) Quantitative detection of microbial genes by using DNA microarrays. *Appl. Environ. Microbiol*. 68: 1425-1430.

Cole, JR, B Chai, RJ Farris, Q Wang, AS Kulam-Syed-Mohideen, DM McGarrell, AM Bandela, E Cardenas, GM Garrity, JM Tiedje (2007) The ribosomal database project (RDP-II): introducing my RDP space and quality controlled public data. *Nucleic Acids Res.* 35 (Database issue): D169-D172.

Cronin MT, RV Fucini, SM Kim, RS Masino, RM Wespi, CG Miyada (1996) Cystic fibrosis mutation detection by hybridization to light-generated DNA probe arrays. *Hum. Mutat* 7: 244-255.

Crump, BC, GW Kling, M Bahr, JE Hobbie (2003) Bacterioplankton community shifts in an arctic lake correlate with seasonal changes in organic matter source. *Appl. Environ. Microbiol*. 69: 2253–2268.

Dahl C, NM Kredich, R Deutzmann, HG Trüper (1993) Dissimilatory sulphite reductase from Archaeoglobus fulgidus: physico-chemical properties of the enzyme and cloning, sequencing and analysis of the reductase genes. *J. Gen. Microbiol*. 139:1817-28.

DeLong, EF, GS Wickham, NR Pace (1989) Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. *Science* 243: 1360-1363.

Demaneche, S, L Jocteur-Monrozier, H Quiquampoix, P Simonet (2001) Evaluation of biological and physical protection against nuclease degradation of clay-bound plasmid DNA. *Appl. Environ. Microbiol.* 67: 293-299.

Dennis, P, EA Edwards, SN Liss, R Fulthorpe (2003) Monitoring gene expression in mixed microbial communities by using DNA microarrays. *Appl. Environ. Microbiol.* 69: 769-778.

DeRisi J, L Penland, PO Brown, ML Bittner, PS Meltzer, M Ray, Y Chen, YA Su, JM Trent (1996) Use of a cDNA microarray to analyse gene expression patterns in human cancer. *Nature Genetics* 14: 457.

de Saizieu, A, U Certa, J Warrington, C Gray, W Keck, J Mous (1998) Bacterial transcript imaging by hybridization of total RNA to oligonucleotide arrays. *Nature Biotechnol*. 16:45–48.

Duineveld, BM, AS Rosado, JD van Elsas, JA van Veen (1998) Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum via denaturing gradient gel electrophoresis and substrate utilization patterns. *Appl. Environ. Microbiol.* 64: 4950-4957.

Edwards, MC, RA Gibbs (1994) Multiplex PCR: advantages, development, and applications. *PCR Methods Appl*. 3: S65–S75.

El Fantroussi, S, H Urakawa, AE Bernhard, JJ Kelly, PA Noble, H Smidt, GM Yershov, DA Stahl (2003) Direct profiling of environmental microbial populations by thermal

dissociation analysis of native ribosomal RNAs hybridized to oligonucleotide microarrays. *Appl. Environ. Microbiol*. 69: 2377-2382.

Farrelly, V, FA Rainey, E Stackebrandt (1995) Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl. Environ. Microbiol*. 61: 2798-2801.

Fox, GE, KR Pechman, CR Woese (1977) Comparative cataloging of l6S ribosomal ribonudeic acid: molecular approach to procaryotic systematics. *Int. J. Syst. Bacteriol.* 27:44-57.

Gans, J, M Wolinsky, J Dunbar (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. *Science* 309: 1387–1390.

Gao, H, ZK Yang, TJ Gentry, L Wu, CW Schadt, J Zhou (2007) Microarray-based analysis of microbial community RNAs by whole-community RNA amplification. *Appl. Environ. Microbiol.* 73: 563–571.

Gentry, TJ, GS Wickham, CW Schadt, Z He, J Zhou (2006) Microarray applications in microbial ecology research. *Microb. Ecol*. 52: 159-175.

Gerry, NP, NE Witowski, J Day, RP Hammer, G Barany, F Barany (1999) Universal DNA microarray method for multiplex detection of low abundance point mutations. *J. Mol. Biol.* 292: 251–262.

Giovannoni, SJ, TB Britschgi, CL Moyer, KG Field (1990) Genetic diversity in Sargasso Sea bacterioplankton. *Nature* (London) 345: 60–63.

Gonzalez, SF, MJ Krug, ME Nielsen, Y Santos, DR Call (2004) Simultaneous detection of marine fish pathogens by using multiplex PCR and a DNA microarray. *J. Clin. Micro* 42: 1414–1419.

Gunderson, KL, XC Huang, MS Morris, RJ Lipshutz, DJ Lokhart, M Chee (1998) Mutation detection by ligation to complete nmer DNA arrays. *Genome Res*. 8: 1142–1153.

Guschin D, B Mobarry, D Proudnikov, DA Stahl, B Rittmann, A Mirzabekov (1997) Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. *Appl. Environ. Microbiol.* 63:2397-2402.

Hacia, JG, JB Fan, O Ryder, L Jin, K Edgemon, G Ghandour, RA Mayer, B Sun, L Hsie, CM Robbins, LC Brody, D Wang, ES Lander, R Lipshutz, SP Fodor, FS Collins (1999) Determination of ancestral alleles for human single-nucleotide polymorphisms using high-density oligonucleotide arrays. *Nature Genet.* 22:164–167.

Ibekwe, AM, CM Grieve, SR Lyon (2003) Characterization of microbial communities and composition in constructed dairy wetland wastewater effluent. *Appl. Environ. Microbiol*. 69: 5060–5069.

Janus, LR, NL Angeloni, J McCormack, ST Rier, NC Tuchman, JJ Kelly (2005) Elevated atmospheric CO2 alters soil microbial communities associated with trembling aspen (Populus tremuloides) roots. *Microb. Ecol.* 50: 102-109.

Jones, JG (1977) The effect of environmental factors on estimated viable and total populations of planktonic bacteria in lakes and experimental enclosures. *Freshwater Biol*. 7:67–91.

Kelly, JJ, S Siripong, J McCormack, LR Janus, H Urakawa, S El Fantroussi, PA Noble, L Sappelsa, BE Rittmann, DA Stahl (2005) DNA microarray detection of nitrifying bacterial 16S rRNA in wastewater treatment plant samples. *Water Res*. 39: 3229-3238.

Kelly, JJ, BK Chernov, I Tovstanovsky, AD Mirzabekov, SG Bavykin (2002) Radical-generating coordination complexes as tools for rapid and effective fragmentation and

fluorescent labeling of nucleic acids for microchip hybridization. *Anal. Biochem.* 311: 103–118.

Kelly, JJ (2003) Molecular techniques for the analysis of soil microbial processes: functional gene analysis and the utility of DNA microarrays. *Soil Sci.* 168:597-605.

Klamer, M, MS Roberts, LH Levine, BG Drake, JL Garland (2002) Influence of Elevated CO_2 on the fungal community in a coastal scrub oak forest soil investigated with terminal-restriction fragment length polymorphism analysis. *Appl. Environ. Microbiol.* 68: 4370–4376.

Koch, R (1876) The etiology of anthrax, based on the life history of Bacillus anthracis. *Beitrage zur Biologie der Pfalanzen* 2: 277-210 (translated by Thomas D. Brock).

Koizumi, Y, JJ Kelly, T Nakagawa, H Urakawa, S El-Fantroussi, S Al-Muzaini, M Fukui, Y Urushigawa, DA Stahl (2002) Parallel characterization of anaerobic toluene- and ethylbenzene-degrading microbial consortia by PCR-denaturing gradient gel electrophoresis, RNA-DNA membrane hybridization, and DNA microarray technology. *Appl. Environ. Microbiol*. 68: 3215-3225.

Konneke, M, AE Bernhard, JR de la Torre, CB Walker, JB Waterbury, DA Stahl (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. *Nature* 437:543-546.

Kuske, CR, SM Barns, JD Busch (1997) Diverse uncultivated bacterial groups from soils of the arid southwestern United States that are present in many geographic regions. *Appl. Environ. Microbiol*. 63: 3614-3621.

Lashkari, DA, JL DeRisi, JH McCusker, AF Namath, C Gentile, SY Hwang, PO Brown, RW Davis (1997) Yeast microarrays for genome wide parallel genetic and gene expression analysis. *Proc. Natl. Acad. Sci. USA* 94: 13057-13062.

Li, LSY, W-T Liu (2003) DNA microarray technology in microbial ecology studies - principle, applications and current limitations. *Microb. Environ.* 18: 175-187.

Li, ESY, JKK Ng, J-H Wu, W-T Liu (2004) Evaluating single-base-pair discriminating capability of planar oligonucleotide microchips using a non-equilibrium dissociation approach. *Env. Microbiol.* 6: 1197–1202.

Liu W, TL Marsh, H Cheng, LJ Forney (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* 63: 4516–4522.

Liu, WT, AD Mirzabekov, DA Stahl (2001) Optimization of an oligonucleotide microchip for microbial identification studies: a non-equilibrium dissociation approach. *Environ. Microbiol.* 3: 619-629.

Liu, W-T., H Guo, J-H Wu (2007) Effects of target length on the hybridization efficiency and specificity of rRNA-based oligonucleotide microarrays. *Appl. Environ. Microbiol*. 73: 73–82.

Loy, A, A Lehner, N Lee, J Adamczyk, H Meier, J Ernst, K-H Schleifer, M Wagner (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl. Environ. Microbiol.* 68: 5064-5081.

Loy, A, C Schulz, S Lucker, A Schopfer-Wendels, K Stoecker, C Baranyi, A Lehner, M Wagner (2005) 16S rRNA gene-based oligonucleotide microarray for environmental monitoring of the betaproteobacterial order “Rhodocyclales”. *Appl. Environ. Microbiol.* 71: 1373–1386.

Ludemann, H, I Arth, W Liesack (2000) Spatial changes in bacterial community structure along a vertical oxygen gradient in flooded paddy soil cores. *Appl. Environ. Microbiol.* 66:754-762.

Lueders, T, MW Friedrich (2003) Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl. Environ. Microbiol.* 69: 320–326.

Macrae, A (2000) The use of 16S rDNA methods in soil microbial ecology. *Brazilian J. Microbiol.* 31: 77-82.

Maskos, U, EM Southern (1993) A study of oligonucleotide reassociation with large arrays of oligonucleotides synthesised on a glass support. *Nucleic Acids Res*. 21: 4663–4669.

Methe, BA, J Webster, K Nevin, J Butler, DR Lovley (2005) DNA microarray analysis of nitrogen fixation and Fe(III) reduction in Geobacter sulfurreducens. *Appl. Envir. Microbiol*. 71: 2530-2538.

Miller, DN, JE Bryant, EL Madsen, WC Ghiorse (1999) Evaluation and optimization of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* 65: 4715-4724.

Mobarry, BK, M Wagner, V Urbain, BE Rittmann, DA Stahl (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl. Environ*. Microbiol 62: 2156-2162.

Moreno-Paz, M, V Parro (2006) Amplification of low quantity bacterial RNA for microarray studies: time-course analysis of Leptospirillum ferrooxidans under nitrogen-fixing conditions. *Environ. Microbiol*. 53:729–737.

Murray, AE, D Lies, G Li, K Nealson, J Zhou, JM Tiedje (2001) DNA/DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. *Proc. Nat. Acad. Sci.* 98: 9853-9858.

Muyzer, G, EC De Waal, AG Uitierlinden (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol*. 59: 695-700.

Ohkuma, M, S Noda, T Kudo (1999) Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. *Appl. Environ. Microbiol*. 65: 4926–4934.

Panicker, G, DR Call, MJ Krug, AK Bej (2004) Detection of pathogenic Vibrio spp. in shellfish by using multiplex PCR and DNA microarrays. *Appl. Env. Microbiol*. 70: 7436–7444.

Parro, V, M Moreno-Paz, E. González-Toril (2007) Analysis of environmental transcriptomes by DNA microarrays. *Environ. Microbiol*. 9: 453–464.

Pease, AC, D Solas, EJ Sullivan, MT Cronin, CP Holmes, SPA Fodor (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Nat. Acad. Sci.* 91: 5022-5026.

Pemov A, H Modi, DP Chandler, S Bavykin (2005) DNA analysis with multiplex microarray-enhanced PCR. *Nucl. Acids Res.* 33: e11.

Peplies, J, FO Glockner, R Amann (2003) Optimization strategies for DNA microarray-based detection of bacteria with 16S rRNA-targeting oligonucleotide probes. *Appl. Env. Microbiol*. 69: 1397–1407.

Pozhitkov, A, B Chernov, G Yershov, PA Noble (2005) Evaluation of gel-pad oligonucleotide microarray technology by using artificial neural networks. *Appl. Environ. Microbiol.* 71: 8663–8676.

Proudnikov, D, A Mirzabekov (1996) Chemical methods of DNA and RNA fluorescent labeling, *Nucleic Acids Res*. 24: 4535–4542.

Purkhold, U, A Pommerening-Röser, S Juretschko, MC Schmid, H-P Koops, M Wagner (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys *Appl. Environ. Microbiol*. 66: 5368–5382.

Raghunathan, A, HR Ferguson, CJ Bornarth, W Song, M Driscoll, RS Lasken (2005) Genomic DNA amplification from a single bacterium. *Appl. Environ. Microbiol.* 71: 3342–3347.

Raskin, L, BE Rittmann, DA Stahl (1996) Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. *Appl. Environ. Microbiol.* 62: 3847–3857.

Rees, WA, TD Yager, J Korte, PH von Hippel (1993) Betaine can eliminate the base pair composition dependence of DNA melting. *Biochem*. 32: 137–144.

Rhee, S-K, X Liu, L Wu, SC Chong, X Wan, J Zhou (2004) Detection of genes involved in biodegradation and biotransformation in microbial communities by using 50-Mer oligonucleotide microarrays. *Appl. Environ. Microbiol*. 70: 4303-4317.

Rich, JJ, RS Heichen, PJ Bottomley, K Cromack, DD Myrold (2003) Community composition and functioning of denitrifying bacteria from adjacent meadow and forest soils. *Appl. Environ. Microbiol*. 69: 5974–5982.

Rooney-Varga, JN, R Devereux, RS Evans, ME Hines (1997) Seasonal changes in the relative abundance of uncultivated sulfate- reducing bacteria in a salt marsh sediment and in the rhizosphere of Spartina alterniflora. *Appl. Environ. Microbiol.* 63: 3895-3901.

Rosch, C, H Bothe (2005) Improved assessment of denitrifying, N_2-fixing, and total-community bacteria by terminal restriction fragment length polymorphism analysis using multiple restriction enzymes. *Appl. Environ. Microbiol.* 71: 2026–2035.

Rotthauwe, JH, KP Witzel, W Liesack (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. *Appl. Environ. Microbiol.* 63: 4704-4712.

Sanguin, H, A Herrera, C Oger-Desfeux, A Dechesne, P Simonet, E Navarro, TM Vogel, Y Moënne-Loccoz, X Nesme, GL Grundmann (2006) Development and validation of a prototype 16S rRNAbased taxonomic microarray for Alphaproteobacteria. *Environ. Microbiol*. 8: 289–307.

Sanguin, H, B Remenant, A Dechesne, J Thioulouse, TM Vogel, X Nesme, Y Moënne-Loccoz, GL Grundmann (2006) Potential of a 16S rRNA-based taxonomic microarray for analyzing the rhizosphere effects of maize on Agrobacterium spp. and bacterial communities. *Appl. Envir. Microbiol*. 72: 4302-4312.

Schena, M, D Shalon, RW Davis, PO Brown (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270:467-470.

Siripong, S, JJ Kelly, DA Stahl, BE Rittmann (2006) Impact of pre-hybridization PCR amplification on microarray detection of nitrifying bacteria in wastewater treatment plant samples. *Env. Microbiol.* 8: 1564-1574.

Small, J, DR Call, FJ Brockman, TM Straub, DP Chandler (2001) Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. *Appl. Environ. Microbiol*. 67: 4708-4716.

Smoot, LM, JC Smoot, H Smidt, PA Noble, M Könneke, ZA McMurry, DA Stahl (2005) DNA microarrays as salivary diagnostic tools for characterizing the oral cavity's microbial community. *Adv. Dent. Res*. 18: 6-11.

Stahl, DA, B Flesher, HR Mansfield, L Montgomery (1988) Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol*. 54: 1079–1084.

Stahl, DA (1995) Application of phylogenetically based hybridization probes to microbial ecology. *Mol. Ecol.* 4: 535-542.

Staley JT, A Konopka (1985) Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial environments. *Ann. Rev. Microbiol.* 39: 321-346.

Starke, EML, JC Smoot, LM Smoot, WT Liu, DP Chandler, HH Lee, DA Stahl (2006) Technology development to explore the relationship between oral health and the oral microbial community. *BMC Oral Health* 6: S10.

Straub TM, DS Daly, S Wunshel, PA Rochelle, R DeLeon, DP Chandler (2002) Genotyping Cryptosporidium parvum with an hsp70 single-nucleotide polymorphism microarray. *Appl. Environ. Microbiol*. 68: 1817-1826.

Suzuki, MT, SJ Giovannoni (1996) Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* 62: 625-630.

Taniguchi, M, K Miura, H Iwao, S Yamanaka (2001) Quantitative assessment of DNA microarrays—comparison with northern blot analyses. *Genomics* 71: 34-39.

Taroncher-Oldenburg, G , EM Griner, CA Francis, BB Ward (2003) Oligonucleotide microarray for the study of functional gene diversity in the nitrogen cycle in the environment. *Appl. Env. Microbiol*. 69: 1159-1171.

Torsvik, V, J Goksoyr, FL Daae (1990) High diversity of DNA of soil bacteria. *Appl. Environ. Microbiol*. 56: 782-787.

Treusch, AH, S Leininger, A Kletzin, SC Schuster, H-P Klenk, C Schleper (2005) Novel genes for nitrite reductase and Amo-related proteins indicate a role of uncultivated mesophilic crenarchaeota in nitrogen cycling. *Environ. Microbiol.* 7: 1985-1995.

Urakawa, H, PA Noble, S El Fantroussi, JJ Kelly, DA Stahl (2002) Single-base-pair discrimination of terminal mismatches by using oligonucleotide microarrays and neural network analyses. *Appl. Environ. Microbiol.* 68: 235-244.

Urakawa, H, S El Fantroussi, H Smidt, JC Smoot, EH Tribou, JJ Kelly, PA Noble, DA Stahl (2003) Optimization of single-base-pair mismatch discrimination in oligonucleotide microarrays. *Appl. Environ. Microbiol.* 69: 2848-2856.

Venter, JC et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* 304: 66-74.

Wagner, M, H Smidt, A Loy, J Zhou (2007) Unravelling microbial communities with DNA-microarrays: challenges and future directions. *Microb. Ecol.* 53: 498–506.

Wang, R-F, ML Beggs, LH Robertson, CE Cerniglia (2002) Design and evaluation of oligonucleotide-microarray method for the detection of human intestinal bacteria in fecal samples. *FEMS Microbiol. Lett.* 213: 175–182.

Ward, BB, D Eveillard, JD Kirshtein, JD Nelson, MA Voytek, GA Jackson (2007) Ammonia-oxidizing bacterial community composition in estuarine and oceanic environments assessed using a functional gene microarray. *Environ. Microbiol.* 9: 2522–2538.

Weber, S, S Stubner, R Conrad (2001) Bacterial populations colonizing and degrading rice straw in anoxic paddy soil. *Appl. Environ. Microbiol.* 67: 1318-1327.

Wick, LM, JM Rouillard, TS Whittam, E Gulari, JM Tiedje, SA Hashsham (2006) On-chip non-equilibrium dissociation curves and dissociation rate constants as methods to assess specificity of oligonucleotide probes. *Nucleic Acids Res.* 34: e26.

Wilson KH, WJ Wilson, JL Radosevich, TZ DeSantis, VS Viswanathan, TA Kuczmarski, GL Andersen (2002) High-density microarray of small subunit ribosomal DNA probes. *Appl. Environ. Microbiol.* 68: 2535–2541.

Wu, L, X Liu, CW Schadt, J Zhou (2006) Microarray-based analysis of subnanogram quantities of microbial community DNAs using whole-community genome amplification. *Appl. Environ. Microbiol*. 72: 4931–4941.

Wu L, D Thompson D, G Li, RA Hurt, JM Tiedje, J Zhou (2001) Development and evaluation of functional gene arrays for detection of selected genes in the environment. *Appl. Environ. Microbiol.* 67: 5780-5790.

Yershov G, V Barsky, A Belgovskiy, E Kirillov, E Kreindlin, I Ivanov, S Parinov, D Guschin, A Drobishev, S Dubiley, A Mirzabekov (1996) DNA analysis and diagnostics on oligonucleotide microchips. *Proc. Nat. Acad. Sci*. 93: 4913-4918.

Zehr, M, MT Mellon, S Zani (1998) New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (nifH) genes. *Appl. Environ. Microbiol*. 64: 3444-3450.

Zehr, JP, BD Jenkins, SM Short, GF Steward (2003) Nitrogenase gene diversity and microbial community structure: a cross-system comparison. *Environ. Microbiol.* 5: 539–554.

Zhang, L, T Hurek, B Reinhold-Hurek (2007) A nifH-based oligonucleotide microarray for functional diagnostics of nitrogen-fixing microorganisms. *Microb. Ecol.* 53: 456-470.

Zhou, JZ (2003) Microarrays for bacterial detection and microbial community analysis. *Curr. Opin. Microbiol*. 6: 288-294.