

SOILS ISSUES

MOLECULAR TECHNIQUES FOR THE ANALYSIS OF SOIL MICROBIAL PROCESSES: FUNCTIONAL GENE ANALYSIS AND THE UTILITY OF DNA MICROARRAYS

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Traditional methods for the analysis of soil processes are based on measuring and modeling the distribution of chemical compounds and determining their transformation rates. These approaches have formed the basis of our understanding of soil biogeochemical processes, and they have demonstrated the fundamental role of microbes in regulating these processes, but they do not provide a complete view of the complexity of the microbial contribution to soil function. Recent research has revealed the tremendous diversity of microorganisms responsible for catalyzing a variety of soil processes. Such functional redundancy within soil microbial communities may have significant impacts on process rates and ecosystem stability. Therefore, the analysis of functional diversity and its dynamics in the environment is essential for understanding the biogeochemistry of soil systems. Until recently, methodological limitations hindered investigation of the relationship between microbial diversity and soil processes. Over the last decade, innovative molecular approaches to the study of natural microbial communities and the functional genes responsible for biogeochemical processes have given us new insight into this relationship. One new approach, DNA microarray analysis, promises to be especially useful for the analysis of these functional genes. (Soil Science 2003;Volume 168:597-605)

Key words: DNA microarrays, 16S rRNA techniques, polymerase chain reaction, T-RFLP, DGGE, RT-PCR.

TRADITIONAL methods for the analysis of soil processes involve measuring the distribution of chemical compounds and determining their transformation rates. For example, the allocation of nitrogen to different pools within the soil can be determined by measuring each of the major nitrogen compounds in a soil sample via standard techniques (Bhagal et al., 2000). Fluxes of compounds between pools can be measured by determining changes in these various pools

over time, by the use of isotopic tracers such as ^{15}N (Geens et al., 1991), or by a variety of biochemical techniques, such as measurement of nitrogen fixation rates by the acetylene reduction assay (Knowles and Barraquio, 1996). These data can then be used to develop models for nitrogen cycling in soils (De Willigen and Neetson, 1985).

These process level approaches have greatly expanded our understanding of the complex array of processes involved in soil function and have demonstrated the fundamental role of microbes in regulating these processes. However, process level measurements do not give us a complete view of the complexity of the microbial contribution to soil function. For a microbially catalyzed soil process, such as nitrogen cy-

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cling, each step in the process is mediated by a metabolically defined group of microorganisms, i.e. a "functional guild" (Taroncher-Oldenberg et al., 2003), and many of these functional guilds consist of phylogenetically diverse collections of microorganisms. For example, several functional guilds are involved in the nitrogen cycle, including denitrifiers, nitrifiers, and nitrogen fixers. Denitrification, the reduction of nitrate to dinitrogen, is a metabolic pathway that is widely dispersed among the prokaryotes, being found mostly in bacteria but also in a few Archaea. Among the bacteria, most of the characterized denitrifiers belong to the Proteobacteria; however, denitrifying bacteria are also well represented among Gram-positive bacteria (Shapleigh, 2002). Nitrification, the biological oxidation of reduced forms of inorganic nitrogen, is also widely distributed across the phylogenetic tree. Lithoautotrophic nitrifiers, which rely on the oxidation of inorganic nitrogen compounds for energy, include two distinct functional guilds within the Gram-negative bacteria that catalyze separate steps in the process of nitrification: the ammonia oxidizers and the nitrite oxidizers. Phylogenetically these two guilds are not closely related (Teske et al., 1994; Purkhold et al., 2000), and, moreover, there is significant phylogenetic diversity within each of these guilds. The ammonia oxidizers include a monophyletic assemblage within the β -subclass of Proteobacteria and a separate assemblage within the γ -subclass of Proteobacteria (Purkhold et al., 2000). Nitrite oxidizers can be found in the α - and γ -subclasses of Proteobacteria (Teske et al., 1994) as well as in the recently described *Nitrospira*-phylum representing an independent line of descent within the domain bacteria (Ehrich et al., 1995). In addition to lithoautotrophic nitrifiers, nitrification can also be carried out by heterotrophic nitrifiers, which are widely dispersed across the phylogenetic tree, being found among the bacteria, fungi, and algae (Focht and Verstraete, 1977; Killham, 1986; Papen et al., 1989). Heterotrophic nitrification is thought to contribute only slightly to the global nitrogen cycle (Brady, 1984), but it may be significant at the local level in certain soils, such as conifer forest soils (Schimel et al., 1984).

The diversity of microorganisms found within functional guilds such as denitrifiers and nitrifiers provides for a high level of functional redundancy within the ecosystem. One possible scenario created by this redundancy is a microbial community containing a number of species each capable of catalyzing a given process. These species may be

active simultaneously, or they may be active under different physical-chemical conditions. Another possible scenario arising from functional redundancy within the ecosystem is that microbial communities in different habitats may be composed of different microbial species yet perform basically the same processes. Therefore, it would be reasonable to hypothesize that functional redundancy could have impacts on rates of biogeochemical cycling, the stability of microbially mediated processes, and the distribution of microbial populations across habitats. The central question, then, is how functional guild composition and diversity are related to ecosystem function. Although the exploration of this question is critical to understanding the microbial ecology and biogeochemistry of soil systems, we know relatively little about the diversity of these functional groups.

Until recently, methodological constraints have made it difficult to assess functional guild diversity effectively (Taroncher-Oldenberg et al., 2003). Culture-based studies, which require the growth of microorganisms in the lab, are severely limited by the fact that less than 1% of environmental microorganisms can be cultured (Amann et al., 1995). Culture-based studies thus provide an extremely restricted view of the diversity of the natural microflora. Advances in molecular techniques, which do not rely on laboratory culture, have led to significant advances in the study of soil microbial communities. The molecular techniques that are commonly used to assess soil microbial communities are summarized in Table 1, and these techniques will be the focus of this review.

16S rRNA TECHNIQUES

Molecular approaches based on small subunit ribosomal RNA (SSU rRNA) have provided a comprehensive phylogenetic framework for the analysis of microbial communities (Amann et al., 1995). 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. Molecular techniques that analyze prokaryotic SSU rRNA (i.e., 16S rRNA) have been applied widely to the study of microbial community structure in soils (Macrae, 2000).

A variety of methods can be used for the analysis of soil microbial communities via 16S rRNA. A phylogenetic inventory of the prokaryotic component of a soil microbial community can be assembled using the Polymerase Chain Reaction (PCR) to amplify all of the 16S rRNA

TABLE 1
Summary of molecular approaches for assessment of soil microbial communities

Scientific objective	Target	Techniques
Assessment of phylogenetic diversity	16S rRNA gene	PCR, cloning, and sequencing T-RFLP or DGGE
Detection of phylogenetic groups	16S rRNA	DNA probes (membrane, in-situ, or microarray hybridization)
Assessment of functional diversity	Functional gene (e. g. amoA)	PCR, cloning, and sequencing T-RFLP or DGGE
Expression of functional genes	mRNA	DNA probes (membrane, in-situ, or microarray hybridization) RT-PCR

genes within a sample, followed by cloning and sequencing this collection of amplified genes. These phylogenetic inventories can be produced for the entire prokaryotic component of a microbial community by conducting the PCR amplification with universal primers designed to amplify all of the 16S rRNA genes within a sample (Borneman et al., 1996; Kuske et al., 1997), or they can be produced for a specific phylogenetic group by using primers designed to amplify the 16S rRNA genes of only that group. For example, Bruns et al. (1999) created a group-specific inventory of ammonia oxidizing bacteria from soil using primers specific for the 16S rRNA of autotrophic ammonia oxidizers. Phylogenetic inventories such as these have revealed much greater diversity in soil microbial communities than was detected by classical culture-based studies, and have thus significantly increased our understanding of soil biological diversity (Becker et al., 2000). However, these inventories are extremely laborious to produce and are, therefore, always incomplete as it is not practical at this stage to sequence every amplicon produced from amplification of an environmental sample (Tiedje et al., 1999).

As an alternative to cloning and sequencing, techniques such as DGGE (Kowalchuk et al., 1998) and T-RFLP (Braker et al., 2001) can be used to analyze the amplicons produced by PCR amplification of DNA extracted from an environmental sample. DGGE and T-RFLP generate profiles for microbial communities based on differences in the 16S rRNA gene sequences of their constituents. These profiles provide insight into differences in microbial population structure between different soil habitats (Ludemann et al., 2000) or changes in soil microbial population structure over time (Duineveld et al., 1998) or

with different experimental treatments (Henckel et al., 2000).

It should be noted that there are some significant limitations to techniques (such as those described above) that are based on PCR amplification of DNA extracted from soil. One assumption of these techniques is that the DNA from all organisms present in the sample will be amplified with the same efficiency. This assumption may not be correct in all cases, and in some cases the process may favor the amplification of DNA from certain organisms. Biases in amplification efficiency can be caused by differences in cell lysis or 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 in phylogenetic surveys and T-RFLP and DGGE profiles (Lueders and Friedrich, 2003), and, thus, it can be problematic in microbial ecological studies (Suzuki and Giovannoni, 1996). A second assumption of PCR-based techniques is that the DNA being amplified has been extracted from living cells. Recent evidence has shown that DNA can persist in soils for significant periods of time by binding to soil particles (Blum et al., 1997) and that this binding can protect free DNA in soils from DNase degradation (Demaneche et al., 2001). Thus, it is possible that some of the DNA amplified by PCR could actually have come from cells that are no longer living in the soil being analyzed. The detection of DNA from dead cells would be a confounding factor in studies of microbial community structure. Therefore, both of these limitations, PCR bias and DNA persistence in soils, should be considered when interpreting results from PCR-based community analyses.

In addition to PCR-based techniques, probe-based methods can be used to detect specific phylogenetic groups of bacteria in soil. DNA probes, which are composed of short segments of DNA, 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). These probes can be hybridized to RNA extracted from a soil sample and can provide information on the presence or absence, as well as relative abundance, of the group targeted by that probe. Ribosomal RNA is a good target for probing technology because active cells contain thousands to tens of thousands of copies of ribosomal RNA per cell, making it a naturally amplified target and often eliminating the need for PCR amplification (Amann and Ludwig, 2000). DNA probes targeting 16S rRNA have been applied widely to the analysis of soil microbial communities (Rooney-Varga et al., 1997; Weber et al., 2001).

16S rRNA-based approaches have provided a wealth of information on soil microbial community composition. However, these approaches have some limitations. Specifically, 16S rRNA-based approaches can indicate which phylogenetic groups of microbes are present in a soil sample, but they provide no information on the metabolic processes being carried out by those groups. In addition, group-specific 16S rRNA approaches may not be suitable for analysis of specific functional guilds if the function is distributed widely over the phylogenetic tree (Braker et al., 2001).

FUNCTIONAL GENES

The analysis of functional genes, i.e., genes encoding enzymes involved in specific metabolic functions, can overcome some of the shortcomings of 16S rRNA-based techniques. Several functional genes have been discovered that are well conserved across phylogenetic groups, including the genes coding for nitrogenase, the enzyme that catalyzes biological nitrogen fixation (Zehr et al., 1995), ammonia monooxygenase, which is involved in nitrification (Rotthauwe et al., 1997), and nitrite reductase, which is involved in denitrification (Braker et al., 1998). That these genes are conserved across phylogenetic groups may make them a more practical choice for the study of these groups than 16S rRNA (Braker et al., 2000). In addition, functional genes often provide a level of resolution below species because functional molecules may experience higher rates of evolutionary change than the 16S rRNA molecule (Braker et al., 2000).

Functional genes can be used to assess the diversity of functional guilds in several ways. Once a functional gene has been identified, the diversity of this gene in different microbial species can be analyzed by PCR-based approaches. Purkhold et al. (2000) used PCR to study the gene that codes for ammonia monooxygenase, the enzyme that catalyzes the first step in the ammonia oxidation pathway (Rotthauwe et al., 1997). The complete gene sequence (designated *amoA*) has been determined for the active site polypeptide of the ammonia oxidizing bacterium, *Nitrosomonas europaea* (McTavish et al., 1993), a type strain isolated from soil (Lewis and Pramer, 1958). Purkhold et al. (2000) amplified a fragment of the *amoA* gene from 17 reference cultures representing all currently recognized species of ammonia oxidizing bacteria (AOB). Sequence analysis of these *amoA* fragments revealed variation in *amoA* sequence among the reference AOB species. When the phylogenetic relationships between the AOB species were determined based on *amoA* sequences, the result was similar but not identical to phylogenetic relationships based on 16S rRNA sequences. These results indicated that *amoA* has strong potential for differentiation of closely related AOB species.

In addition to the analysis of reference cultures, the diversity of functional genes can be assessed directly from the environment. Specific PCR primers can be used to amplify the target gene from DNA extracted from environmental samples. This mixture of functional gene amplicons can be used to create a clone library, and individual clones can be sequenced and compared. Rotthauwe et al. (1997), working with DNA extracted from soil and sewage samples, created such a clone library using PCR primers specific for the *amoA* gene. Sequencing of 47 of these clones revealed that the *amoA* gene amplification was highly specific for AOB, and comparison of *amoA* sequences allowed fine scale resolution of closely related AOB species. Following a similar strategy, Zehr et al. (1998) used PCR primers specific for conserved regions of the *nifH* gene that codes for a protein component of nitrogenase, the enzyme that catalyzes biological nitrogen fixation. The researchers amplified the *nifH* gene from marine samples, and the PCR amplicons were cloned and sequenced. The results indicated that the ocean samples contained more diverse nitrogen-fixing microbial populations and more diverse habitats for nitrogen fixers than observed previously by classical microbiological techniques (Zehr et al., 1998).

The diversity of environmentally derived functional gene amplicons can also be assessed by profiling techniques such as T-RFLP (Scala and Kerkhoff, 2000; Braker et al., 2001) and DGGE (Henckel et al., 2000). For example, Horz et al. (2000) used *amoA*-specific primers to amplify a section of the *amoA* gene from DNA isolated from environmental samples and examined these amplicons using T-RFLP. The T-RFLP profiles obtained from the environmental samples enabled the researchers to rapidly assess the complexity of the ammonia-oxidizing community in the environmental samples.

There has also been development of DNA probes targeting functional genes. Smith and Tiedje (1992) developed a set of probes targeting the dissimilatory nitrite reductase gene (*nir*), which is involved in denitrification. These denitrifier probes were successful in detecting variants of the *nir* gene in aquifer microcosms. Ka et al. (1994) successfully detected the gene coding for degradation of the herbicide 2,4-dichlorophenoxyacetic acid in soil samples by membrane hybridization of a DNA probe for the gene to DNA extracted from the samples.

DNA Microarrays

The application of DNA probes is generally carried out using membrane-based (Sahm et al., 1999) or *in situ* (Amann et al., 1995) hybridization techniques. These techniques can be used for probes targeted to the 16S rRNA or to various functional genes. However, because these hybridization techniques severely limit the number of probes that can be applied simultaneously, thus limiting the amount of information that can be acquired, the scope of such probe studies is often limited.

As an alternative to conventional hybridization techniques, DNA microarrays offer a much higher probe capacity. DNA microarrays generally consist of a set of nucleic acids that are spotted and covalently bound to some solid support, such as a glass slide (Small et al., 2001). On a typical DNA microarray, hundreds to tens of thousands of nucleic acids can be spotted within a very small surface area, and this array of nucleic acids can be hybridized simultaneously (Wilson et al., 2002). In addition to higher probe capacity, microarrays also offer the advantages of increased speed of detection, low cost, and the potential for automation (Shalon et al., 1996). DNA microarray technology has been used for characterizing entire genomes (Dong et al., 2001) as well as for measuring gene expression (Schena et al., 1995).

DNA microarray technology has great potential for characterizing microbial communities and their function in the environment. However, the application of DNA microarrays to the assessment of microbes in the environment does pose a number of technical challenges, including optimization of probe-target specificity and quantification of target genes. Recent work has demonstrated that these technical challenges can be overcome. Urakawa et al. (2002, 2003), working with the probe Nsom 0653, which targets a region of the 16S rRNA that is specific for the ammonia oxidizing genus *Nitrosomonas*, demonstrated that excellent probe-target specificity could be achieved on a DNA microarray by analysis of thermal dissociation profiles. Cho and Tiedje (2002), working with the nitrate reductase gene *nirS*, demonstrated that target genes in biological samples could be quantified using reference DNAs to normalize hybridization signals.

Microarrays containing DNA probes targeting 16S rRNA have recently been applied to the assessment of microbes in the environment (Koizumi et al., 2002; Loy et al., 2002). Recent work has demonstrated that microarrays can be used for the direct detection (i.e., without the need for PCR amplification) of 16S rRNA extracted from soil samples (Small et al., 2001; El Fantroussi et al., 2003). Based on the potential advantages of DNA microarrays and recent technical advances, it is likely that the application of 16S rRNA-targeted DNA microarrays to environmental systems will increase dramatically in the near future.

Recently, microarrays targeting functional genes have also been developed and applied to the analysis of soil microbial communities. Taroncher-Oldenberg et al. (2003) designed a DNA microarray that included oligonucleotide (70-mer) probes targeting different variants of the genes involved in the nitrogen cycle (*amoA*, *nifH*, *nirK*, and *nirS*). They extracted DNA from sediment samples from two stations in the Choptank River in Maryland. This extracted DNA was amplified and fluorescently labeled by PCR, and this labeled DNA was hybridized to the microarray. Hybridization patterns on the microarray differed between the sediment samples from the two stations in the Choptank River that differed in salinity, inorganic nitrogen, and dissolved organic carbon. These differences in hybridization patterns reflected differences in the distribution of variants of these functional genes, implying important differences in the composition of the nitrifier and denitrifier communities.

Functional Gene Expression

The studies discussed above have assessed the presence of functional genes by PCR-based techniques. These studies can contribute to our understanding of the diversity and distribution of members of various functional guilds. It is also possible to assess the expression of functional genes in environmental samples by the detection of mRNA. Since mRNA is a critical intermediate in gene expression, and since mRNA has a short half-life, detection of mRNA specific for a gene is a strong indicator that the gene is being actively converted to protein (Nogales et al., 2002). In addition, the number of mRNA transcripts is correlated with level of activity (Weinbauer et al., 2002). Several different techniques have been employed to assess functional gene expression by detecting mRNA.

RT-PCR is an effective tool for mRNA detection. In RT-PCR, mRNA is used as a template to synthesize a complementary strand of DNA (cDNA), and this cDNA is then amplified by PCR. Recently, Zani et al. (2000) utilized reverse transcriptase PCR (RT-PCR) to detect the expression of nitrogenase genes in lake samples. They extracted RNA from the samples, this RNA was amplified by RT-PCR, and the amplicons were cloned and sequenced. The sequences of these clones indicated which variants of the nitrogenase genes were being expressed in the sample. They also amplified the nitrogenase genes from DNA extracted from the same samples by conventional PCR and, again, cloned and sequenced the amplicons. The sequences of these clones indicated which variants of nitrogenase genes were present in their samples. Combining these two data sets indicated which groups of nitrogen-fixing microorganisms were present in the samples and which of the groups were actively expressing their nitrogenase genes. This powerful analysis method has also been applied to sediments (Nogales et al., 2002) and could be an excellent technique for the analysis of functional guilds in soils.

Detection of mRNA can also be done via DNA probes and conventional hybridization techniques. Stapleton et al. (1998) used DNA probes and mRNA extracted from environmental samples to monitor the expression of several microbial catabolic genes in soil collected from bioremediation sites. Probes targeted to mRNA can also be applied in a DNA microarray format. Thus far, the uses of microarrays to monitor gene expression has been confined largely to analysis of expression patterns in single organisms, usually

Escherichia coli (Oh and Liao, 2000; Wei et al., 2001). However, recent work has employed DNA microarrays for the detection of expression in complex microbial communities. Dennis et al. (2003) built a DNA microarray that included 64 functional genes from a variety of organisms. In their study sample, RNA was extracted and then converted to cDNA by reverse transcription, and the cDNA was fluorescently labeled and hybridized to the microarray. The microarray was able to detect expression of a number of the genes in pure microbial cultures, constructed mixed microbial communities, and in pulp mill bioreactor samples. This technique shows great promise for the monitoring of gene expression in soils.

CONCLUSIONS

Molecular techniques have revolutionized the field of soil microbiology over the last 20 years. Application of these techniques to the study of 16S rRNA genes has created a comprehensive framework for microbial phylogeny and has dramatically expanded our understanding of soil microbial diversity. Recent functional gene studies have already contributed to our understanding of functional guild diversity, and functional gene analysis techniques will ultimately allow for effective monitoring of the expression of these genes in soil. An exciting new technology, DNA microarrays, has already demonstrated its utility as a format for the application of both 16S rRNA and functional gene targeted DNA probes, and the further development of DNA microarrays for environmental monitoring will undoubtedly continue. Because of their high probe capacity, DNA microarrays offer the potential to build complex arrays that integrate monitoring of both 16S rRNA and functional genes, which will enable us to examine both the phylogenetic and functional components of soil microbial communities. Therefore, molecular techniques, and DNA microarrays in particular, should help to revolutionize our understanding of the complex role that microorganisms play in soil processes.

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