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ECO-BIOLOGICAL SOIL ASSESSMENT: ANALYTICAL APPROACHES THROUGH MOLECULAR METHODS

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Introduction

Soil organisms facilitate key ecosystem processes such as nutrient cycling and organic matter decomposition, and also influence soil porosity and water infiltration through the production of bacterial polymers and fungal hyphae that hold soil particles together to form aggregates [1, 2]. Rhizosphere microorganisms can greatly influence the ability of a plant to acquire macro- and micronutrients. This may occur as a direct consequence of symbiotic associations between plant roots and microorganisms such as arbuscular mycorrhizal fungi, through symbiotic and associative interactions such as the fixation of N_2 by the root-associated bacteria *Rhizobium* spp., *Azospirillum*, *Azotobacter*, or *Beijerinckia* spp. [3], or through trophic interactions, such as the excretion of nutrients by the microbial feeders protozoa and nematodes [4]. Through these interactions, soil organisms powerfully affect crop growth and yield. Soil management strategies should aim to support and enable soil biota to carry out these key ecosystem functions to assure long-term soil fertility and sustained crop production. In order to understand better how soil management practices influence soil biota and their interactions with plant roots, appropriate methods are urgently needed to detect, enumerate and characterize them.

Analytical approaches

A longstanding challenge in soil microbiology has been developing effective methods to describe both the diversity and function of soil microbial populations. Population diversity encompasses the genetic variability within a species, the number (richness) and relative abundance (evenness) of species, and functional groups within communities [5]. The problem is that the species concept, derived from plant and animal community ecology, does not translate well to microbial populations. As yet, there is no satisfactory species concept for bacteria and fungi [6], making it rather difficult to characterize these populations in ecologically meaningful ways. The advent of molecular ecology has not resolved, but rather complicated the debate as more becomes known about the lateral transfer of genetic elements between bacteria in the environment. Diversity at the ecosystem scale includes the variety of processes occurring, the complexity of interactions between organisms and the number of trophic levels represented in the population.

It is well known that traditional culturing methods detect only a small fraction of the extant microbial diversity in soil [7]. Molecular techniques employing bulk soil DNA extraction and cloning with or without polymerase chain reaction (PCR) amplification with “universal”

primers for rRNA genes often reveal the presence of populations that are not recovered by traditional culturing [6]. However, populations detected by molecular methods such as clone libraries and DNA fingerprinting, may still not correspond to populations responsible for significant biogeochemical processes *in situ*, especially when these populations constitute less than 1% of the total community. Use of PCR primers specific to known representatives may help to facilitate their detection [8]. However, knowing the taxonomic identity or phylogenetic affiliation of a cloned sequence, does not mean that we will know the function of the organism *in situ*. In addition, simple presence of an organism in a sample does not necessarily mean it is active. This latter point has spurred development of RNA-based techniques and stable isotope labelling to help pinpoint those members of a community that are most active under a given set of environmental conditions. Application of molecular methods must be based on the recognition that soil organisms are physiologically and phylogenetically diverse. Hence, a polyphasic approach, one that employs culturing, activity measures and a combination of molecular techniques, is likely to yield a more comprehensive understanding of the function of soil biology in cropping systems. Discussion of the variety of methods used for polyphasic characterization of soil communities is beyond the scope of this presentation; instead I will focus on recent molecular methods that are being used to characterize soil microbial composition and, in some cases, the functions of select members of the soil community. The relationship between many of the below-mentioned techniques and how each is used in microbial community analysis is shown in Figure 1. Amplification of rRNA genes forms the basis for most of these techniques. Figure 1 also includes reference to more traditional approaches used in soil microbiology and indicates how they support and relate to the new molecular approaches.

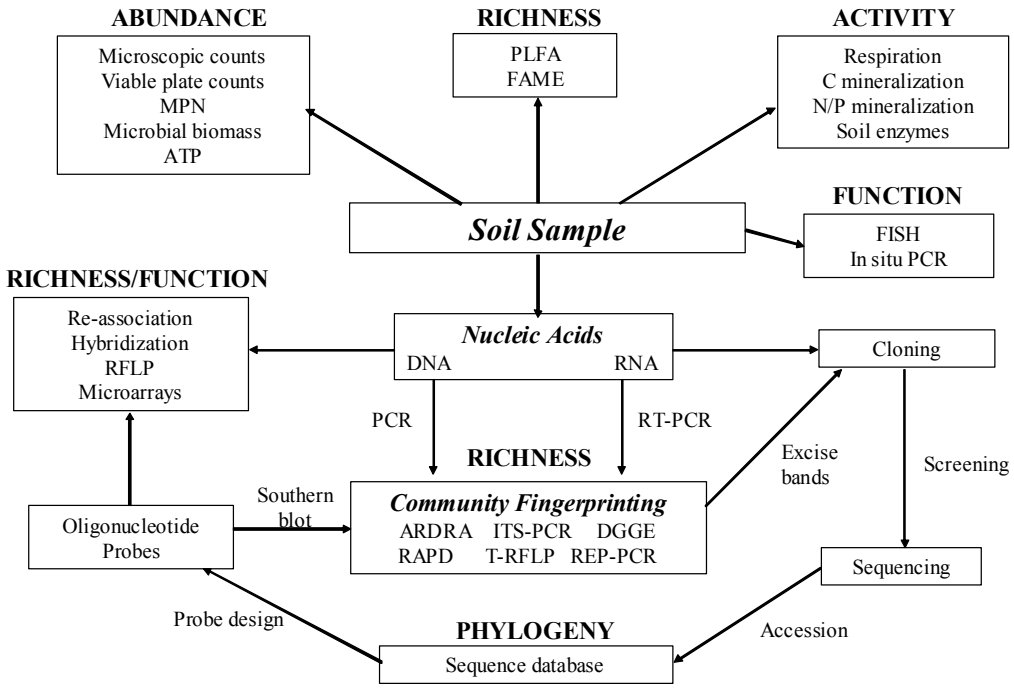

Figure 1. Overview of analytical methods

Molecular methods

Phospholipid fatty acids (PLFA)

Phospholipid fatty acid (PLFA) analysis is a technique for studying the soil microbial community that does not rely on the ability to culture the organisms. It is a non-selective method, wherein phospholipid fatty acid composition of the soil is analyzed by gas chromatography (GC) [9, 10]. PLFAs are the basic components of cell membranes and are metabolized rapidly when cells die in the soil. Consequently, extraction of phospholipids from soil samples provides information about the microbial communities present [10, 11].

The PLFA profile is used as a 'fingerprint' of the soil community. Since phospholipid-linked branched fatty acids are characteristic of bacterial origin, lipids can also be used to indicate specific subgroups within the community and physiological status of those populations. For example, sulfate reducers, methane-oxidizing bacteria, mycorrhizal fungi and actinomycetes have unique lipid signatures. Further, environmental variation can induce changes in certain PLFA components, such as the ratio of saturated to unsaturated fatty acids, ratio of trans- to cis-monoenoic unsaturated fatty acids and the proportion of cyclopropyl fatty acids, thus heralding changes in the microbial community. In addition, PLFA profiles may contain information concerning the dynamics of larger groups of organisms such as eukaryotes. However, typical fatty acids (e.g. polyenoic fatty acids) found in eukaryotes are less distinguished in comparison to a number of fatty acids found almost exclusively in bacteria [9].

This technique has been used to study microbial community changes during vermi-composting and composting [12, 13] and has more recently been combined with stable isotope labeling to track changes in microbial community structure resulting from specific carbon-13 (^{13}C) additions to soil. Analysis of ^{13}C labeled PLFAs generated by introducing substrate labeled with stable isotopes offers a way to define the groups of organisms using those substrates. Thus, PLFA analysis of the soil microbial community can be coupled with ^{13}C isotope tracer analysis to measure the microbial community's response to the addition of glucose, utilized by many microorganisms, or toluene, used by a small subset of micro-organisms. The hypothesis is that ^{13}C from glucose will become incorporated into a large number of the PLFAs extracted from the soil community, as compared to a small set of PLFAs that would become labeled from ^{13}C toluene [14]. Hence, PLFA analysis has the potential to investigate the diversity of microbial communities in various soil samples.

Nucleic acid approaches

Molecular methods have revolutionized the study of microorganisms and their activities. The ability to extract DNA or ribosomal RNA (rRNA) from cells contained within soil samples, and their direct analysis in hybridization experiments or use in polymerase chain reaction (PCR) amplification experiments [15] has allowed us to detect the presence of a vast diversity of microbes previously unimagined. The application of these techniques to studying microbial communities has overcome the limitations inherent with traditional enrichment and isolation techniques, thereby enabling detection of organisms yet to be cultivated. Before nucleic acids can be characterized, they must be extracted from the soil matrix. Extraction of nucleic acids from soil may have a considerable amount of associated bias that must be acknowledged when interpreting the results of subsequent analyses. Once extracted, DNA may be analyzed directly or specific DNA target sequences may be amplified by PCR and the resulting PCR products further characterized. Or, in the case of rRNA, complementary DNA (cDNA) is first derived

from the sample by reverse transcription and the cDNA produced is subsequently analyzed (see Figure 1).

The aim of many molecular approaches is to describe population diversity as described by taxon richness. Due to bias in both DNA and rRNA extraction and PCR amplification, it is difficult, if not impossible to assess the abundance of different taxa using these approaches [16]. In addition, these methods are still not refined to the point where function can be unambiguously assigned to different taxa. Hence, molecular methods should be used in concert with other approaches to achieve a more holistic understanding of the structure and function of the soil microbial community.

Extraction of nucleic acids (DNA/RNA)
A variety of methods to extract nucleic acids from soils of varying texture have been developed and these have recently been summarized by Bruns & Buckley [17]. There are two main approaches to nucleic acid extraction: cell fractionation and direct lysis. In cell fractionation, intact microbial cells are extracted from the soil matrix. After extraction, the cells are subsequently lysed and the DNA separated from the cell debris. In the direct lysis methods, microbial cells are lysed directly in the soil and then the nucleic acids are separated from the soil matrix. The main concerns when choosing a suitable protocol are extraction efficiency, obtaining a sample that is representative of the resident community, and obtaining an extract free of contaminants that could interfere with either PCR or probe hybridization.

Extraction efficiency of both cell fractionation and direct lysis procedures can be assessed by direct microscopy using vital stains, where extracted soil is examined for intact microbial cells. Alternatively, for assessing extraction efficiency using direct lysis procedures, soil samples may be spiked with a known quantity of DNA and then the recovery of added DNA assessed.

Obtaining a sample that is representative of the resident community also presents a challenge. Microbes in soil are often more difficult to lyse than cells grown in pure culture. In addition, cell walls of different organisms lyse with varying efficiencies. Cell walls of high G+C Gram-positive bacteria are often challenging to lyse, whereas those of Gram-negative bacteria lyse more readily. Hence, recovered DNA or rRNA may contain an artificially greater amount of DNA derived from Gram-negative bacteria.

Co-extraction of contaminants, such as humic and fulvic acids, is a common problem. Such contaminants interfere with PCR amplification to the point where the reaction fails entirely. Hybridization experiments are likewise affected. Several methods have been suggested to eliminate or reduce contaminants. One approach is to run the DNA extract through a post-PCR DNA clean-up kit, such as the Qiaquick® PCR purification kit (Qiagen, Chatsworth, CA). These kits are normally designed to remove salts from post-PCR amplification reactions, however, they also work to reduce contaminants in DNA extracts and have eliminated PCR inhibition in many instances in my laboratory (unpublished data). An alternative method is to subject the nucleic acid extract to an additional washing step with dilute EDTA or pass the extract through a Sephadex G-75 column [17]. It must be kept in mind that all manipulations of the extract can lead to loss of material and hence sparsely represented members of the community may be lost from subsequent analyses. In addition, all post-extraction clean-up procedures add extra expense and processing time, thus reducing the number of samples that can be analyzed within the scope of any experiment. DNA extraction protocols for specific use with compost have been developed by La Montagne et al. [18] and Howeler et al.[19].

Commercial soil DNA/RNA extraction kits based on direct lysis by bead-beating [20-22], such as the FastDNA® SPIN Kit for Soil and the FastRNA® Pro Soil-Direct Kit (Qbiogene, Inc., Carlsbad, CA) and the Ultraclean™ and PowerSoil™ DNA isolation kits (MoBio Laboratories, Solana Beach, CA) have recently become available. The DNA/RNA extracted is of high molecular weight and of sufficient quality to be used in PCR or nucleic acid hybridization experiments for most soils. The PowerSoil™ DNA isolation kit (MoBio Laboratories) has been specifically recommended, by the manufacturer, for use with compost.

Direct analysis of nucleic acid extracts

DNA:DNA re-association kinetics

The genetic complexity or genome size of a microbial community can be assessed by re-association of community DNA. Using this procedure, Torsvik et al. [23] estimated that the community genome size in undisturbed organic soils was equivalent to 6,000-10,000 *E. coli* genomes. Polluted soils contained 350-1500 genome equivalents, whereas culturing methods produced less than 40 genome equivalents. Such experiments, along with epifluorescence microscopy, are what substantiate the lack of abundance and diversity captured with culturing methods.

When DNA is denatured by either heating or use of a denaturant (e.g., urea), the double helix structure is lost as the two strands, held by hydrogen bonds between complementary base pairs (A:T and G:C), come apart. When the denaturant is removed or the temperature lowered, complementary strands re-anneal. When genome complexity is low, the time it takes for all strands to find their complement is brief. As complexity increases, the time it takes for complementary strands to re-anneal increases. Experimentally, this is referred to as a $C_o t$ curve, where C_o is the initial molar concentration of nucleotides in single-stranded DNA and t is time. This measure reflects both the total amount of information in the system (richness and number of unique genomes) and the distribution of that information (evenness and abundance of individual genomes) [6]. Yet, it provides no information on identity or function of any member of the microbial community.

Nucleic acid hybridization

Nucleic acid hybridization involves hybridizing a discrete fragment (a probe) of DNA or RNA to a target sequence. The probe is generally labeled with a radioisotope or fluorescent molecule and the target sequence is bound to a nylon membrane. A positive hybridization signal is obtained when complementary base pairing occurs between the probe and the target sequence. This signal is visualized by exposing the membrane to auto-radiographic film after removal of any unbound probe. The type of probe used and how the probe is labeled determine the applications of nucleic acid hybridization techniques. For example, oligonucleotide probes (up to 30 nucleotides long) may be used under very stringent conditions which resolve single base-pair mismatches but have limited sensitivity of detection due to the constraint on the number of labels that may be attached to the probe. In contrast, larger DNA fragments may be labeled to high specific activity but it is difficult to control hybridization conditions sufficiently to guarantee 100% stringency. Table 1 gives examples of probes that may be used to pose specific questions about presence of selected organisms in a soil community. There are several web sites that offer probe design (http://www.arb-home.de/) and (http://rdp.cme.msu.edu/html/) and information about probes that have already been designed for specific purposes (http://www.microbial-ecology.net/probebase/).

Techniques based upon nucleic acid colony hybridizations (colony blotting) have particular value in rapidly screening clone libraries. Nucleic acid probes can also be fluorescently labeled and hybridized to identify organisms *in situ* using the fluorescent *in situ* hybridization (FISH) technique. These protocols have been described extensively in reviews by Amann et al. [24, 25]. The advantage of FISH is the ability to visualize and identify organisms on a microscale in their natural environment [26]. Such techniques have enormous potential for the study of soil microbial ecology. However, binding of fluorescent dyes to organic matter resulting in non-specific fluorescence is a common problem that may be exacerbated in high organic matter soils. In addition, membrane hybridizations may be inhibited by contaminants co-extracted with soil microbial DNA.

Table 1. Hybridization probes targeting different levels of resolution in the analysis of soil microbial communities.

Name	Sequence (5' to 3')	Target gene	Community target	Reference
EUB338	GCTGCCTCCCGTAGGAGT	16S rRNA	Most Eubacteria	[27]
UNIV 1389b	ACGGGCGGTGTGTACAAA	18S rRNA	Most Eukarya	[28]
UNIV 1389c	ACGGGCGGTGTGTGCAAG	16S rRNA	Archaea	[28]
BET42a	GCCTTCCCACTTCGTTT	23S rRNA	β-Proteo-bacteria	[29]
beta-AO233	AGCTAATCAGRCATCGG	16S rRNA	Ammonium oxidizers	[30]

Microarrays

Microarrays represent an exciting new development in the analysis of microbial communities. The technology is based on nucleic acid hybridization and confocal laser scanning microscopy (CLSM). The main differences between past protocols and microarrays are that: an oligonucleotide probe, rather than the DNA or RNA target, is immobilized on a solid surface; and hundreds of probes, bound in a miniaturized matrix, can be tested at the same time [31]. A fully-developed DNA microchip could include a set of probes encompassing virtually all natural microbial groupings and thereby serve to simultaneously monitor the population structure at multiple levels of resolution [6]. Such an array would allow for high throughput of samples. The details of chip construction and types of arrays can be found in Ekins & Chu [32]. Microarrays hold exciting potential for the exploration of soil microbial communities and for pathogen detection in soil and/or compost. However, the sensitivity of detection through use of this technology, which borders on 10^5 copies of the target sequence, is still orders of magnitude away from where it needs to be for use as a robust monitoring or detection tool. As probe design and specificity and detection systems improve, microarrays will play a larger and larger role in soil microbial community analysis.

Restriction fragment length polymorphism (RFLP) analysis

In this technique, total DNA purified from soil is cut with a restriction endonuclease (often *Eco*R1 or *Hin*dIII). The variation (polymorphisms) in the length of resulting DNA fragments is visualized by running the DNA fragments on an electrophoretic gel and staining the gel with ethidium bromide followed by illumination under UV light. These polymorphisms, visualized as a 'DNA fingerprint' are then used to differentiate between soil communities.

Discrimination between communities is often difficult because of the large number of fragments generated. RFLP is rarely used on its own for diversity studies. It is most commonly used in conjunction with Southern blotting followed by nucleic acid hybridization with an oligonucleotide or gene probe (see Hybridization Section above) or in the restriction digestion of amplified ribosomal genes in techniques such as terminal restriction fragment length polymorphism (T-RFLP) analysis as described below.

Cloning
There are two main ways in which DNA sequence information is obtained from environmental samples: DNA probe hybridization (see above) and chain termination sequencing of cloned (either shot-gun or PCR-amplified) DNA templates. Cloning involves isolating DNA from the soil, ligating the DNA into a vector, such as pGEM-T (Promega), and transforming the vector into a host bacterium, such as *E. coli*, thereby generating a recombinant DNA library. Once a clone library is obtained, DNA inserts contained in the clones can be sequenced at a DNA sequencing facility. The clone library can also be screened for biological activity expressed directly in *E. coli* [33] or probed for sequences of interest in a genomics-type application. This approach circumvents the need to culture microorganisms from environmental samples, and provides a less biased sampling of the genetic diversity of those environments.

Recently, it became possible to clone large fragments (100–300 kb) of DNA into bacterial artificial chromosome (BAC) vectors [34]. BAC vectors are low-copy plasmids that can readily maintain large DNA inserts. When Rondon et al. [34] analyzed their BAC libraries, they found sequences homologous to the low-G+C, gram-positive *Acidobacterium*, *Cytophagales*, and *Proteobacteria*. They also identified clones that expressed lipase, amylase, nuclease, and hemolytic activities. Hence, the library could be used for both phylogenetic studies and as a tool for natural product discovery. Such metagenomic libraries are powerful tools for exploring soil microbial diversity and will form the basis for future genomic studies that link phylogenetic information with soil microbial function [33].

Partial community analyses – polymerase chain reaction-based assays

Polymerase chain reaction (PCR)
PCR involves the separation of a double-stranded DNA template into 2 strands (denaturation), the hybridization (annealing) of oligonucleotides (primers) to the template and then the elongation of the primer-template hybrid by a DNA polymerase enzyme [15, 35]. The potential target genes for PCR are many and varied, limited only by available sequence information. The primers most frequently used for soil ecological studies are designed to target specific DNA fragments, such as 16S or 18S rRNA genes or functional genes; repetitive sequences, such as REP (Repetitive Extragenic Palindromic) sequences; or arbitrary primers, e.g. randomly amplified polymorphic DNA (RAPD). Some oligonucleotides that are commonly used in PCR-fingerprinting are listed in Table 2.

Both RAPD primers and repetitive sequence primers can provide a fingerprint or profile for any particular target genome. In general, the annealing temperature at which the primers [36] are used determines whether single primers such as RP01 or BOXA1R or paired repetitive element (rep) primers such as ERIC (Enterobacterial Repetitive Intergenic Consensus sequences) and REP actually target specific sequences or act as RAPD primers [37]. A major limitation of PCR-fingerprinting with RAPD-type primers is that the fingerprint patterns obtained may vary due to subtle variations in PCR conditions [37-39]. Hence, standardization

of all aspects of PCR both within and between laboratories is critical, particularly if resulting data are to be shared.

Table 2. PCR primers targeting different levels of resolution in the analysis of soil bacterial communities.

Name	Sequence (5' to 3')[1]	Target gene	Reference
27F 1392R	AGAGTTTGATCMTGGCTCAG ACGGGCGGTGTGTRC	16S rRNA	[40]
1490F 132R	TGCGGCTGGATCACCTCCTT CCGGGTTTCCCCATTCGG	16S – 23S rRNA internal transcribed spacer (ITS)	[41] [42]
amoA-1F *amoA*-2R	GGGGTTTCTACTGGTGGT CCCCTCKGSAAAGCCTTCTTC	*amo*A Ammonium mono-oxygenase	[43]
PolF PolR	TGCGAYCCSAARGCBGACTC ATSGCCATCATYTCRCCGGA	*nif*H Nitrogenase promoter	[44]
A189 A682	GGNGACTGGGACTTCTGG GAASGCNGAGAAGAASGC	*pmo*A Methane mono-oxygenase	[45]
ERIC 1R ERIC 2	ATGTAAGCTCCTGGGGATTCAC AAGTAAGTGACTGGGGTGAGCG	Repeated elements	**[46]**
REP1R REP 2	IIIICGICGICATCIGGC ICGICTTATCIGGCCTAC	Repeated elements	**[47]**

[1]B = g, T or C; K = G or T; N = A, C, g or T; R = A or g; S = G or C; Y = C or T; I = inosine

By far the most common targets for characterization of microbial communities are the ribosomal RNA gene sequences (*rrn* operon). These are the small subunit (SSU) rRNA genes, 16S in prokaryotes or 18S in eukaryotes; the large subunit (LSU) rRNA genes, 23S in prokaryotes or 28S in eukaryotes; or the intervening transcribed spacer regions between the SSU and LSU gene sequences. Other defined targets are genes that code for ecologically significant functions, such as genes involved in nitrogen fixation, e.g. *nif*H, which encodes the iron protein of nitrogenase reductase [44, 48]; *amo*A which codes for ammonium monooxygenase, a key enzyme in nitrification reactions [43, 49]; and *nir*S which codes for nitrite reductase, a key enzyme in denitrification reactions [50, 51].

In any study where PCR will be employed, sources of bias must be considered. The main sources of bias in amplification of soil community DNA are: use of very small sample size (typically 500 mg of soil), which may represent only a small fraction of the whole soil community; preferential amplification of some DNA templates over others due to the greater ease of binding of DNA polymerase to some sequences than others; generating chimeric sequences composed of double stranded DNA where each strand was derived from a different organism (often a consequence of too many cycles in the PCR); and, for amplification of the rRNA genes, the fact that many bacteria contain multiple copies of these operons (e.g., *Bacillus* and *Clostridium* species contain 15 *rrn* copies), hence sequences from such species

will be over-represented among the amplification products. In PCR-based approaches, such biases need to be taken into account when interpreting results.

Electrophoresis of nucleic acids
Amplified PCR products are most often visualized by running samples out on an electrophoretic gel and then staining the gel with ethidium bromide. Analysis of the amplification products is based on the presence and pattern of DNA bands in the gel matrix. Agarose is the most popular medium for electrophoretic separation of medium and large-sized nucleic acids. Agarose has a large working range, but poor resolution. Depending upon the agarose concentration used, nucleic acids between 0.1 kilobase (kb) and 70 kb in size can be separated. Polyacrylamide gels can also be used. Polyacrylamide is the preferred matrix for the separation of proteins, single-stranded DNA fragments up to 2000 bases in length or double-stranded DNA fragments of less than 1 kb. The resolving power of polyacrylamide gels is such that they distinguish the electrophoretic mobility of macromolecules on the basis of configuration in addition to the more commonly exploited characteristics of size, charge and G+C content. This shape dependent mobility forms the basis of a suite of techniques that exploit inter- and intra-strand nucleotide interactions and can be used to rapidly screen culture collections for very fine scale sequence differences that are beyond the capacity of routine hybridization methods. These techniques include single strand conformation polymorphism (SSCP) (intra-strand) [52], denaturing gradient gel electrophoresis (DGGE) [53], temperature gradient gel electrophoresis (TGGE) [54] and heteroduplex mobility assays (HMA) [55, 56] (all inter-strand). Because the electrophoretic mobility of nucleic acids using these techniques is highly sequence dependent, these techniques offer considerable flexibility to researchers for any screening program aimed at detecting genetic diversity.

Denaturing or temperature gradient gel electrophoresis (DGGE/TGGE)
DGGE and TGGE are identical in principle. Both techniques impose a parallel gradient of denaturing conditions along an acrylamide gel. Double stranded DNA (dsDNA, homoduplex) is loaded and, as the DNA migrates, the denaturing conditions of the gel gradually increase. In DGGE, the denaturant is generally urea; in TGGE it is temperature. Because native dsDNA is a compact structure, it migrates faster than partially denatured DNA. The sequence of a fragment determines the point in the gel at which denaturation will start to retard mobility. Sequence affects duplex stability by both percentage G+C content and neighboring nucleotide interactions (e.g. GGA is more stable than GAG).

While the power of DGGE and TGGE to detect amplicon diversity within a single gel is very high, the sensitivity of the technique makes comparisons between gels very difficult. These techniques are therefore of greatest use in a preliminary screening to aid recognition of sample diversity. As with all electrophoretic techniques, the resolving power is limited by the number of bands capable of "fitting" and being resolved on one gel. In practice, no more than 100-200 distinct sequence types may be resolved despite the single base-pair sensitivity. However, DGGE and TGGE have an advantage over restriction fragment length polymorphism (RFLP) analyses in offering greater sensitivity and requiring less manipulation of the sample. They are also amenable to use with mixed samples. DGGE and TGGE are now frequently being applied in microbial ecology to compare the structures of complex microbial communities and to study their dynamics [16, 57-59]. An important advantage that DGGE analysis has over T-RFLP (see below) is that amplicons of interest that are resolved on a DGGE gel can be excised from the gel, reamplified, cloned and sequenced, thereby obtaining taxonomic and/or phylogenetic information about amplifiable members of the soil community. However, Ovreas

et al. [16] have shown that, compared with reassociation kinetics, DGGE vastly underestimates the extant diversity.

Terminal restriction fragment length polymorphism (T-RFLP) analysis
T-RFLP analysis exploits rapid community DNA extraction methods, PCR, RFLP, the resolution of polyacrylamide gels and automated sequencing technology, and 16S rRNA gene sequence database information to determine key bacterial groups present in environmental samples. Liu et al. [60] used this technique to characterize microbial diversity within bioreactor sludge, aquifer sand and termite guts. Schloss et al. [61] and Tiquia et al. [62] have applied T-RFLP successfully to follow population changes during the initial stages of composting and in fully composted livestock manures, respectively. This technique need not be restricted to studying the 16S rRNA gene. T-RFLP can be used as a quick screen with any gene to look at differences between communities in environmental samples. The real advantage of this technique is that it simplifies data to a manageable number of bands, i.e., reducing an amplified ribosomal DNA restriction analysis (ARDRA) pattern to one visualized fragment per organism. Resulting terminal restriction fragments (TRF) are sized and comparisons between samples made by use of similarity matrices and clustering analysis. The main drawback of the use of this approach is the inability to further characterize TRFs or obtain sequence information.

Stable isotope probing (SIP)
Nucleic acid methods have recently been coupled with stable isotope labeling and detection to provide a cultivation-independent means of linking the identity of bacteria with their function in the environment [63, 64]. Either soil is incubated after addition of a ^{13}C-labelled substrate or a plant is labeled with ^{13}C-CO_2 and rhizosphere soil sampled after labeling. Soil DNA or RNA is then extracted and subjected to density gradient centrifugation. The ^{13}C-labelled DNA produced during the growth of metabolically distinct microbial groups that can grow on the ^{13}C-enriched carbon source or ^{13}C-labeled root exudates can thus be separated from ^{12}C-DNA. The ^{13}C-labelled DNA can be subsequently characterized taxonomically and functionally by sequence analysis and gene probing [65-67].

DNA sequencing
Significant progress, particularly in working with environmental samples, has been made since Sanger et al. [68] first described the dideoxy chain termination method for DNA sequencing. The advent of fluorescent dyes, improvements in gel matrix technology, cyclic sequencing using a PCR machine, use of lasers and automated gel analysis now allow up to 1600 bases of sequence to be generated or deduced in a single reaction. Manual sequencing is extremely time-consuming and is very rarely performed in laboratories nowadays. It is more cost effective to send your DNA and primer to a commercial sequencing facility.

The usefulness of DNA sequencing to environmental studies lies in determining gene sequences of unique members of the soil community of interest for use in developing more specific primers and gene probes. Gene sequences are submitted to and maintained within various databases such as the Ribosome Database Project (http://rdp.cme.msu.edu/html/) or GenBank (http://www.ncbi.nlm.nih.gov/). The Ribosome Database Project II (Cole et al., 2003) contains over 100,000 prokaryotic sequences with updated on-line analyses. This database is maintained by the Center for Microbial Ecology (CME) at Michigan State University. Continued development of databases through DNA sequencing is essential and is a pre-requisite to good primer and probe design.

Software for similarity analyses
The successful application of DNA amplification and electrophoresis to population studies and to systematics relies heavily on the correct interpretation of the banding patterns observed on electrophoretic gels. There are a number of software packages on the market that will compare and score 'PCR- fingerprint' banding patterns and produce similarity values for a given set of samples. Such packages include BioNumerics and GelCompar (Applied Maths, Kortrijk, Belgium), Diversity Database and Molecular Analyst (BioRad, Hercules, CA, USA), RFLP Scan (Scanalytics, Billerica, MA, USA) and Dendron (Solltech, Inc., Oakdale, IA, USA). An advantage of using analysis programs such as Bionumerics, GelCompar, Molecular Analyst or Diversity Database is that fingerprints of communities generated from the use of several different markers can be combined [69]. Generating a combined fingerprint in this way increases the robustness of similarity analyses based on PCR-fingerprints because it reduces the impact that one or two minor band differences has on the production of similarity matrices. Analytical support for the analysis of T-RFLP data is available on the RDPII web site (http://rdp.cme.msu.edu/html/analyses.html).

Biosensors and marker gene technologies
Introduced marker genes are now being used more frequently in the study of microbial ecology. One such gene that has attracted considerable attention in rhizosphere studies is *gfp*, which encodes Green Fluorescent Protein (GFP). One reason for its popularity is that there is no GFP background activity in plants or the bacteria and fungi that interact with them, thereby making *gfp* an excellent target gene that can be introduced into selected bacterial strains and used to study plant-microbe interactions [70-72]. Basically, *gfp* is transposed into either the chromosome or plasmid of a bacterial strain where it is subsequently maintained. Various transposon constructs have been made, which differ in the type of promoters or terminators used and some contain repressor genes such as *lac*I for control of *gfp* expression. Once key populations in a soil or compost are known and isolates are obtained, they can be subsequently marked in this way in order to track them and assess their functions and interactions in soil and the rhizosphere. This is a promising technology for future work.

Level of resolution
Genes change, that is, they acquire fixed mutations over time. The number of differences between 2 homologous sequences reflects both the evolutionary rate of the sequences and the time separating them, that is, how long it has been since they had a common ancestor. Consequently, different sequences need to be selected to resolve variation at different taxonomic levels. In general, non-coding DNA evolves faster than transcribed DNA, since it is under no selection pressure to remain unchanged; therefore, intergenic spacer regions evolve more rapidly than other sequences. Next is the "wobble" position of protein coding genes and slowest to change are the structural rRNA genes [73].

The information that can be obtained from molecular characterization also depends on the analysis technique. 16S rRNA gene sequencing can aid in assigning species into genera and can be used for determining relationships between genera, but the information is frequently unable to resolve differences between closely related species [74]. To overcome this limitation, one could additionally employ the information contained within IGS regions either by sequencing or by RFLP to further discriminate between closely related species.

Sequence differences between DNA fragments (not necessarily genes) can be very quickly assessed using DGGE, TGGE, or SSCP techniques or by a combination of RFLP, Southern

blotting and nucleic acid hybridization with specific probes. Sequence differences between genes that encode enzymes can be analyzed by use of multi-locus enzyme electrophoresis (MLEE) techniques. The level of resolution required, coupled with study aims, will largely guide the choice of technique used for a given study (see Figure 1).

To add value to the study of soil community ecology, a technique must be robust, that is, yield specific information about communities at the level of resolution required; it must be rapid and allow high throughput in order for the large number of samples needed for landscape studies to be processed with moderate effort.

Applications

We have come a long way in developing our understanding of microbial ecology, but have many milestones yet to meet. Molecular tools offer unparalleled opportunities to characterize bacteria in culture and directly from field soils or composts. These tools are allowing us to ask questions at much larger geographic scales than have been possible previously. We are now able to examine such issues as how microbial populations vary across soil types and climatic zones, in association with plant roots and between various plant species, and in response to soil management or soil pollution. Molecular approaches also provide improved tools for seeking new inoculant consortia that may provide benefit in cropping systems. Genotypes that enjoy high representation in the soil population are likely to be competent saprophytes, adapted to site conditions. Pre-adapted strains that are also highly effective and genetically stable would then be excellent target organisms for future inoculants.

Not surprisingly, there is still much to be done. In a recent issue of Science magazine, soil has been dubbed 'The Final Frontier' [75]. Modern molecular techniques developed to study of microbial populations, such as T-RFLP [60, 76] and DGGE [59], allow access to the very large proportion of organisms that are present in the soil and which remain unculturable under laboratory conditions. Other techniques, such as metabolic fingerprinting [77] and RFLP analysis of radio-labeled amplified *nif*H [78], *amo*A [43, 49], *nir*S [50], and *pmo*A sequences [45, 79] will allow us to target, with high specificity, organisms or groups of organisms at different taxonomic levels. These should prove to be useful detection tools in ecological studies. These types of technical developments open new horizons of research and applications that will allow a far more complete and less biased view of bacterial biodiversity in soil and compost.

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