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Molecular and functional diversity in soil micro-organisms

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Abstract

Traditional approaches to the study of microbial diversity have relied on laboratory cultivation of isolates from natural environments and identification by classical techniques, including analysis of morphology, physiological characteristics and biochemical properties. These approaches provide information on fine-scale diversity but suffer from bias, resulting from the media and cultivation conditions employed, and from the inability to grow and isolate significant proportions of natural communities in the laboratory. An alternative approach is the amplification of ribosomal RNA and functional genes from nucleic acids extracted directly from environmental samples, with subsequent analysis by 'fingerprinting' methods or by sequencing and phylogenetic analysis. This approach avoids the need for laboratory cultivation and has provided major insights into species and functional diversity of bacterial and archaeal populations. This article reviews molecular approaches to the characterisation of prokaryote diversity in natural environments, their more recent application to fungal diversity and the advantages and limitations of molecular analyses.

Introduction

Micro-organisms are essential for the functioning and sustainability of all natural ecosystems but are frequently ignored due to their small size and consequent methodological difficulties in detecting cells and assessing their activity. Methodology has also severely limited characterisation and analysis of species composition, species diversity and structure of microbial communities. The central role of microorganisms in ecosystem processes, including biogeochemical cycling of nutrients and biodegradation, makes it both unwise and dangerous to ignore any aspect of their ecology. Analysis of diversity is particularly important when considering the ability of ecosystems to respond to changing environmental conditions, the need for conservation of the microbial gene pool and the links between diversity, ecosystem processes, functional and physiological diversity, resilience and sustainability. This article describes the major recent advances in techniques for the characterisation of microbial diversity which provide the potential to address these important issues.

Several approaches are available for assessment of microbial diversity (Atlas, 1984). For those groups with distinctive morphological characteristics, direct observation may be appropriate. This approach is, however, very limited, particularly for analysis of diversity in complex environments, such as soil, and when quantification of measures of diversity is required. Until recently, the only approaches available for assessment of diversity were those based on laboratory cultivation of organisms from environmental samples. Typically, cell suspensions were prepared from environmental samples, dilution series constructed and samples plated onto solid medium. Following incubation, pure cultures obtained from isolated colonies were identified using well-established phenotypic characters. This approach provides information on which species are present, while the relative abund-

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ance of different organisms can be used to determine measures of diversity, e.g. diversity indices, evenness and dominance. The potential limitations of this approach are widely acknowledged and accepted (Torsvik, 1996). Separation of biomass from particulate material will vary between species and with growth form (cells, spores, mycelia), introducing bias. More significantly, it is impossible to design growth media and cultivation conditions that are suitable for all members of the community. Selection of particular groups is inevitable and some organisms may not be able to grow under any of the cultivation conditions utilised. Others will be viable but non-culturable or dormant, requiring lag periods that lead to their being out-competed during incubation or prevent detectable growth within realistic incubation periods. These biases are accentuated when quantification of diversity is required and, for filamentous fungi, are further complicated by difficulties in quantifying and relating biomass derived from hyphae and spores, and consideration of which proportion of mycelial biomass is active. Identification using traditional methods, based on phenotypic characteristics, also raises problems.

Despite these considerable and well-accepted limitations, cultivation-based assessment of microbial diversity has been employed, largely because of the lack of acceptable alternatives, and advances have been made. For example, identification can be accelerated by automated methods, e.g. Biolog, and broad scale measures of diversity, e.g. phospholipid fatty acid (PLFA) profiling (Tunlid et al., 1989), fatty acid methyl ester (FAME) profiling (Cavigelli et al., 1995), DNA hybridisation and re-association (Torsvik et al., 1990), have overcome some of the limitations of cultivation-based techniques. Some of these provide information on functional diversity, e.g. Biolog, which can otherwise be obtained by physiological characterisation of laboratory isolates. They do not, however, enable the fine-scale analysis of diversity that is required to determine the importance of species diversity, and of particular species, on ecosystem function.

Molecular approaches

An alternative approach, which removes many of the above limitations, is the analysis of genes within environmental samples. These genes may be functional genes, i.e. those coding for proteins performing particular metabolic reactions of relevance to ecosystem processes. However, most applications have analysed genes encoding the small subunit (SSU) of ribosomal RNA. Analysis of 16S rRNA genes is now widely used for analysis of bacterial populations, and analysis of 18SS rRNA genes and internal transcribed spacer (ITS) regions is increasingly being used to analyse fungal populations. Ribosomal rRNA genes are ideal for this purpose in that they possess regions with sequences conserved between all bacteria or fungi, facilitating alignment of sequences when making comparisons, while other regions exhibit different degrees of variation, enabling distinction between different groups. These differences provide the basis for a phylogenetic taxonomy and enable quantification of evolutionary differences between different groups. Discrimination of bacteria, using 16S rRNA gene sequences, is greater than that that for fungi, using 18S rRNA sequences, but finer scale information may be obtained by analysis of ITS regions.

The presence of regions of rDNA sequence with different degrees of conservation enables the identification of sequences that are common to all bacteria or fungi, or to specific phylogenetic groups, sometimes to the level of species. These sequences may then be used to design primers for the specific amplification, using the polymerase chain reaction (PCR), of rRNA genes belonging to particular groups or to design specific probes for these groups. These primer sequences provide the basis for analysis of species in natural populations. Two approaches may be adopted, the first based on PCR amplification of rRNA genes and the second involving *in situ* detection of rRNA within cells.

Analysis of amplified genes

The first stages in the analysis of rRNA genes in an environmental sample are cell lysis and extraction of DNA, after which DNA is purified to remove material inhibitory to subsequent enzymatic reactions. PCR amplification is then carried out, using primers specific to the microbial groups of interest. Amplification generates a population of rRNA genes, or gene fragments, of equal size, determined by the particular primers used. This population of gene fragments is considered to be representative of the natural microbial population.

Most information, and fine scale discrimination between groups, is obtained by cloning the amplified rRNA genes and sequencing members of the clone library. Comparison of sequences with those in

databases determines which phylogenetic groups are present and, in many cases, enables more detailed identification. This approach is particularly useful for studies of bacteria, as 16S rRNA databases are now extensive and comprehensive. They contain sequences of large numbers of laboratory cultures and also of clones obtained from a range of environments, which are not represented in laboratory cultures. Fungal databases are increasing in size, but are currently less extensive and informative, except for phylogenetic groups that have been the subject of detailed taxonomic study Phylogenetic analysis provides information on the numbers of sequences falling within different groups and may identify novel groups with no sequences of cultured organisms. Sequences obtained in this manner may be used to refine existing primers and probes, increasing their sensitivity and specificity, and to design primers and probes for novel groups of uncultured microorganisms. Finally, if sufficiently large numbers of clones are sequenced, estimates may be obtained of the relative abundance of different groups.

More rapid analysis is achieved using fingerprinting techniques. The most commonly used technique in 16S rRNA studies has been denaturing gradient gel electrophoresis (DGGE, Muyzer et al., 1993), which separates products of the same size, but different sequence, by chemical denaturation. Following staining of gels, banding patterns may be used to compare communities, or to compare the same community following perturbations, and band intensities may be used for semi-quantification of relative abundance (McCaig et al., 1999, 2001). In addition, bands may be excised and genes amplified and sequenced for fine scale analysis. A similar approach is adopted in temperature gradient gel electrophoresis (TGGE), where denaturation results from high temperatures (Felske et al., 1998). A number of fingerprinting techniques involve restriction analysis of PCR products, including terminal restriction length polymorphism (tRFLP, Liu et al., 1997) and amplified ribosomal DNA restriction analysis (ARDRA) (Øverås and Torsvik, 1998). In some cases, database information may be used to predict the banding patterns generated using these techniques by particular rRNA gene sequences, providing some information on the identity of organisms present. PCR-based methods, such as competitive PCR (Jansson and Leser, 1996) and real-time PCR (Heid et al., 1996) are also used to quantify gene copies, and hence cell number or biomass.

Fluorescence in situ *hybridisation (FISH)*

Sequence information can also be used to design and construct fluorescently labelled oligonucleotide probes specific for particular microbial groups. These can then be used to detect cells *in situ* using FISH (Amann et al., 1990). This technique relies on the large number of ribosomes within cells, particularly actively growing cells, which greatly increases sensitivity in comparison to detection of single gene copies. This approach is obviously of great value in determining the location and spatial organisation of different organisms and signal strength may be related to cell activity (but see below). It is also useful in providing supporting evidence for quantitative estimates of relative abundance obtained using PCR-based approaches.

Benefits and limitations

The benefits of molecular techniques have been indicated above, the most important being the ability to characterise diversity without the requirement for laboratory cultivation. In addition, molecular identification, where possible, is more rapid than cultivation-based methods and is becoming the method of choice for commercial identification of many microbial groups.

Molecular techniques avoid problems that necessarily arise from selective media and cultivation conditions and the non-culturability of many cells, but introduce other sources of bias. Lysis efficiency will vary between microbial groups, between spores and mycelia and between regions of mycelia of different ages, influencing DNA extraction yields. Subsequent purification of DNA will reduce yield, potentially also with bias. PCR amplification and use of primers can lead to differences in the proportions of different rRNA genes and sequencing errors, formation of chimeric sequences and introduction of errors during cloning can influence results. *In situ* probing requires semi-permeabilisation of cells, conditions for which will vary within mixed populations. There are also problems associated with molecular-based quantification. Sequence abundance may not necessarily relate directly to cell numbers, for example, through variation in rDNA copy number between different organisms. Considerable care must be exercised when attempting to quantify following PCR amplification and estimation of relative abundance from DGGE gels must be considered semi-quantitative only. As we will see below, phylogenetic information can provide some information on the physiology of organisms, through

knowledge of common characteristics of cultivated organisms belonging to particular groups. However, this is not always the case, limiting interpretation of species diversity assessments with regard to ecosystem function.

It is possible to test for many of these potential problems and, in general, cross-referencing and use of several independent techniques indicate that molecular approaches provide true reflection of diversity and community structure within natural assemblages. However, the molecular approach is in its infancy and the importance of these limitations is not yet fully understood, and development of solutions is at an early stage. Molecular techniques have provided enormous advances in our understanding of natural bacterial populations. In the following sections, these will be exemplified by studies of 'total' bacteria and of a more specialised group, ammonia oxidising bacteria, followed by examples of molecular characterisation of 'total' fungal populations. Approaches being developed to assess the mechanisms controlling and driving diversity, and the significance of microbial diversity for ecosystem processes will then be discussed.

Bacterial diversity in soil

Analysis of total bacterial populations can be achieved by PCR amplification of extracted DNA using universal primers, designed to amplify 16S rRNA genes of bacteria and archaea. The following examples typify application of this approach to soil bacterial populations.

In the first, Borneman et al. (1996) analysed 16S rRNA gene clone libraries from East Amazonian soil. Of 124 clones sequenced, 98.4% fell within the *Bacteria* the remainder being mitochondrial DNA. There was considerable diversity, with only 40 of sequences being duplicated, and approximately 25% of sequences fell within five groups that could not be assigned to previously characterised groups within the *Bacteria*. Although they did not obtain isolates, the sequences from extracted DNA differed significantly from those of cultured organisms typically isolated from soil. For example, only 19% and 0.8% of sequences were representative of bacilli and actinomycetes, respectively, and no sequences were found that were representative of pseudomonads, agrobacteria, *Alcaligenes* or *Flavobacterium*. Similar results were found in a study of Brazilian forest and pasture soils (Borneman and Triplett, 1997), with no duplicate sequences in 100 clones analysed, 98 and 2 of which belonged to the *Bacteria* and *Archaea* respectively. No sequence was identical to sequences in databases and 19 were only distantly related to known sequences, suggesting the possibility of new kingdoms. Differences were also found between pasture and forest soils. Kuske et al. (1997), investigating a cinder site (arid, thermal soils with low organic carbon) and a sandy loam soil also found no duplicates in clone libraries and no identity to sequences in databases. Of 56 sequences analysed, 20 were affiliated with known bacterial groups but the remainder fell within five distinct novel groups, different at the division level, which also included novel sequences from other studies. RFLP analysis of 600 cultured isolates from these soils gave no identity with the novel divisions.

While taking into account the potential biases and limitations of 16S rDNA-based techniques, the significance of these findings is remarkable. They suggest that a substantial proportion (sometimes greater than 50%) of soil prokaryotes belong to groups with no representative in laboratory culture. The lack of cultured representatives, and our consequent ignorance of their metabolic and physiological characteristics, prevent meaningful speculation regarding their role in biogeochemical cycling and other environmental processes.

More recent studies have attempted to use molecular techniques, including DGGE analysis of 16S rDNA, to quantify species diversity, evenness and dominance, and to determine the effects of soil management regimes and treatments on prokaryotic diversity (McCaig et al., 1999a, 2001). The high levels of diversity within individual soil samples make it difficult to detect differences between treatments and collector's curves indicate that, even when sequences are grouped at the 95% level of homology, analysis of several hundred clones is required before adequate coverage is achieved.

Diversity of ammonia oxidising bacteria

Autotrophic ammonia oxidising bacteria were traditionally placed in five genera, on the basis of a limited number of phenotypic characters. Analysis of 16S rRNA genes of pure cultures (Head et al., 1993, Purkhold et al., 2000) defined one genus, *Nitrosococcus oceanus*, in the λ-proteobacteria and two genera, *Nitrosomonas* and *Nitrosospira*, in the β-proteobacteria

which comprise the majority of strains found in the environment. Ammonia oxidisers grow slowly in the laboratory, with low biomass yields, and isolation and identification are difficult. As a consequence, traditional approaches provided little information on their natural diversity and 16S rDNA-based molecular techniques have proved to be invaluable. Primers specific to β-proteobacterial ammonia oxidisers have been used to amplify and analyse 16S rRNA genes from DNA extracted from a range of environmental samples and the results are typical of those obtained for other microbial groups with specialised function.

Sequence diversity within ammonia oxidisers appears to be high, with very few duplicate 16S rDNA sequences in clone libraries. Sequences frequently differ by only a few bases, possibly through sequencing and cloning errors, but the length of sequence amplified is relatively small (approximately 300 bases) and sequencing of larger stretches or of functional genes (e.g. ammonia monooxygenase) reveals greater diversity. Initial phylogenetic analysis of culture and environmental clone sequences suggested the existence of seven phylogenetic clusters within the *Nitrosomonas* and *Nitrosospira* genera (Stephen et al 1996), but subsequent analysis indicates the possible existence of more (Purkhold et al., 2000). The different clusters are associated with different environments. For example, *Nitrosomonas europaea* cluster 6 and *Nitrosospira* clusters 1 and 5 are obtained from marine environments and *Nitrosospira* clusters 2, 3 and 4 from terrestrial and freshwater environments. Clusters 1 and 5 have no representative in laboratory culture and we cannot therefore predict their distinctive physiological characteristics or ecological role. It is possible, but unlikely, that they are not ammonia oxidisers. Genera previously considered to be restricted to particular environments are now recognised as being more widely distributed. For example, *Nitrosospira* was considered to be absent from marine environments but is now routinely detected.

The ammonia oxidiser type strain *Nitrosomonas europaea* originally isolated from soil, has been the subject of the majority of physiological studies of ammonia oxidation, but sequences related to this strain are rarely encountered and *Nitrosospira* appears to be more important in these environments. This highlights the potential dangers of studies based on laboratory cultivation and the advantages of molecular techniques, which enable us to determine which enrichments and isolates are representative of natural populations. Sequences amplified from directly extracted DNA and from cultures derived from the same environmental samples (Smith et al., 2001) differ significantly in their distribution between ammonia oxidiser clusters and duplicate sequences are found more frequently in enrichment cultures. This indicates laboratory selection of particular groups, but a greater understanding of biases associated with molecular techniques is required before deciding which is the correct picture of natural diversity.

Characterisation of diversity and cluster divisions enabled assessment of the influence of environmental conditions on ammonia oxidiser diversity and community structure. For example, differences have been observed in ammonia oxidiser communities in polluted sediment beneath fish cages (McCaig et al., 1999b), in marine aggregates (Phillips et al., 1999) and in acid soils (Bruns et al., 1999; Stephen et al., 1998). These studies demonstrate the ability of 16S rRNA methods to detect changes in community composition with changes in environmental conditions. They are providing the basis for an understanding of the mechanisms controlling diversity and, importantly, the relationship between species diversity, functional diversity and their impact on process rates.

Fungal studies

Molecular techniques have been used to detect and analyse fungal populations for several years and their specific application to mycorrhizal fungi will be described elsewhere in this volume. In many cases, these techniques have been used to characterise strains isolated on laboratory media or material extracted from hyphae. Studies such as those described above, in which fungal populations are characterised by analysis of DNA extracted from bulk samples, are more rare. This approach is, however, now increasing and several examples will be described. The major advantage is the ability to assess diversity without the requirement for laboratory growth and isolation of fungi. Some studies are based on small subunit rRNA (18S rRNA) but this region possesses less useful information than 16S rRNA genes of prokaryotes. To obtain greater specificity, others have analysed ITS regions. A major limitation, in comparison with bacterial studies, is the relative lack of sequence information in databases but this problem is decreasing and molecular identification, where possible, is more rapid.

Kowalchuk et al., (1997) used nested PCR and DGGE analysis to characterise fungal populations in-

fecting *Ammophila arenaria* which are believed to lead to degeneracy in marram stands. Primers were originally designed to amplify 18S rRNA genes from pure cultures and were not specific, but there was little interference from other soil eukaryotic populations in the relatively specialised environment represented by plant roots. Distinct bands were produced from pure cultures of a range of fungi, but band position in itself does not necessarily provide phylogenetic information, which requires excision and sequencing of bands. Sequences of representative bands from DGGE analysis of healthy and degenerating roots (field samples) were sometimes identical or closely related to those of cultivated fungi, including some isolated from marram roots, but others were only distantly related to known fungal species or showed no clear affiliation with known fungal groups. Also, sequences of several fungi commonly isolated from these systems were not detected and there were indications of differences in populations from roots of healthy and degenerate marram stands.

Primer sets with greater specificity were developed by Smith et al. (1999) for analysis of fungal populations in the wheat rhizosphere. Two of the primer sets were suitable but with one, EF4–EF3, showing bias towards basidiomycetes and zygomycetes and a second, EF4–fung5, with bias towards ascomycetes. The 39 clones obtained using the EF4–fungs primers contained no basidiomycete sequence, while 22 clones derived from EF4–EF3 primers contained only two representatives of the ascomycetes. The 61 clones contained representatives of 24 species but in many cases sequences showed little similarity to those in databases, preventing identification. This reflects the relative paucity of sequence information in the databases at the time, but may also indicate the existence of novel groups that have not yet been cultivated. Banding patterns obtained by TGGE analysis of PCR products indicated bias and differences were found between fungal communities in bulk and rhizosphere soils in microcosms and following incubation of soil for 5 and 10 days. This approach, therefore, provides a means of studying changes in fungal communities in soil with the potential for identification by sequencing bands of interest from TGGE or DGGE gels.

Borneman and Hartin (2000) also developed fungal specific primers for analysis of two avocado grove soils, considered to be suppressive and conducive to infections by *Phytophthora cinnamomi*. Both primer sets were specific and successfully amplified 18S rRNA from pure cultures but differences were found in environmental clones sequences amplified by these primers and those of Smith (1999), emphasising the need for care in interpretation of results and potential primer bias and selectivity. To compare fungal population in disease-suppressive and disease-conducive soils, cloned 18S rRNA genes were sorted into 10 clone types and relative abundances determined for each soil. Significant differences were seen between soils, with four clone types absent from the disease-conducive soil. Comparison of sequences representative of each clone group with those in databases indicated that the most dominant genera differed significantly from those genera isolated using cultivation-based techniques.

Although primer specificity is desirable, in certain situations with low non-fungal diversity, less specific primers may be useful. For example, Vainio and Hantula (2000) analysed fungi colonizing Norway spruce stumps. The lack of non-fungal eukaryotic contaminants enabled comparison of wood colonisers isolated in pure culture and those detected by molecular techniques. In many cases, sequences obtained from extracted DNA and pure cultures were identical, but differences were found in 4 of 17 samples, suggesting selection of non-dominant strains during cultivation. 18S rRNA genes provide good coverage of fungal taxa but do not enable fine scale analysis. This can be obtained by analysis of ITS sequences and primers have been developed for amplification of ITS regions from extracted DNA. For example, Viaud et al. (2000) analysed ITS regions amplified from DNA extracted from soil and from fungi cultivated from the same soil samples. Amplified genes from 67 cultures and 51 clones were grouped on the basis of RFLP patterns and 58 representative ITS sequences were compared with those in databases. At the level of precision used in this study, only one of the cultivated fungi was represented in the clone library. In addition, all cultivated fungi were ascomycetes, except for one basidiomycete, while clone libraries contained a wider range of taxa.

Links between species diversity, physiology and ecosystem processes

Molecular techniques, as illustrated above, are capable of characterising the diversity, species composition and community structure of microorganisms in natural environments. While this information is of value, the most important questions concern the significance

of this diversity for ecosystem function. Molecular studies have demonstrated differences in species composition in different environments and differences associated with different treatments. To understand the basis of these differences, we need to determine the characteristics of different components of natural communities and their respective ecosystem functions. Molecular techniques can contribute to such questions and, for unculturable organisms are essential. Examples of a number of approaches are given below.

Analysis of functional gene diversity

Although species composition can be characterised by analysis of 16S rRNA gene sequences, this may not tell anything about the functional diversity of the community, or even which functions can be carried out. Metabolic processes of ecological relevance may be distributed among several phylogenetic groups, while a single group is likely to be capable of performing many functions. This is particularly pertinent when considering, for example, transformations of organic carbon that will be carried out by many heterotrophic microorganisms. Genes for many processes have now been sequenced in a number of organisms. It is, therefore, possible to design primers for the amplification of specific genes, which can then be analysed, as described above for 16S rRNA genes. The diversity of gene sequences within an environment can be characterised and differences in relative abundance of functional genes belonging to different phylogenetic groups can be assessed. For some groups, and some functional genes, groups formed on the basis of functional gene analysis will be related directly to phylogenetic groups. However, this will not always be the case, for example, when functional genes are plasmid-borne or where genes have been transferred horizontally. An important issue in this approach is the choice of functional gene. The success of an organism in a particular environment will depend on a range of properties, rather than the product of a single gene. Functional genes must therefore be chosen with care.

Analysis of RNA

RNA may be used in two ways as an indicator of activity. Detection of mRNA for a particular gene can be used to assess gene expression and determine the response of a particular function to changes in environmental conditions. This may also provide information on which species are responding, if functional and taxonomic genes are related. This approach has been limited by the short half-life of mRNA and difficulties in extracting sufficient quantities for analysis before degradation.

The second approach assumes that more active and faster growing organisms possess more ribosomes and, consequently, higher levels of ribosomal RNA. Studies on the influence of growth rate on ribosomal levels have been restricted to a few organisms and the relationship is not simple, varying between different organisms with, for example, different rates of ribosome degradation following cessation of activity through starvation. Nevertheless, (semi-) quantification of RNA is used to indicate levels of activity of different components of a community and analysis of RNA provides significantly greater sensitivity, as cells typically contain tens of thousands of ribosomes. RNA may be extracted from soil and hybridised with specific, hierarchical probes to determine the relative abundance of different members of the community (Purdy et al., 1997). Alternatively, reverse transcriptase-PCR (RT-PCR) may be used to amplify rRNA genes using RNA, rather than DNA, as a target. Comparison of DGGE profiles following PCR amplification of rRNA genes from both extracted DNA and RNA distinguishes active and inactive populations on the basis of ribosome abundance and can be used to determine which members of the community become active under particularly conditions.

Stable isotope probing

Stable isotope probing (SIP) has enormous potential for determining which components of a microbial community are capable of incorporating a particular substrate, labelled with a heavy isotope. SIP was first used to determine which methanol-utilisers were active in soil. Following incubation of soil with ^{13}C-labelled methanol, DNA was extracted and ^{13}C and ^{12}-DNA separated by density gradient centrifugation. ^{13}C-labelled DNA will be derived only from organisms that have grown and assimilated methanol into DNA and these organisms can be detected by amplification of 16S rRNA genes or functional genes from ^{13}C-DNA, followed by sequence analysis. This approach therefore enables identification of organisms actively metabolising specific compounds *in situ*, with no requirement for laboratory growth. Radajewski et al. (2000) used this technique to distinguish methanol utilisation by two bacterial groups, the α-proteobacterial and *Acidobacterium* lineages, detecting sequences from uncultured organisms. One current

limitation of this approach is its sensitivity, requiring levels of growth that enable incorporation of sufficient DNA for separation and detection of ^{13}C-DNA, although sensitivity can be increased by analysis of RNA, rather than DNA. In common with all methods involving substrate amendment, sampling and analysis must be carried out before significant turnover of the substrate occurs.

FISH combined with microautoradiography

The use of FISH, using rRNA probes, has been described above for cell detection in environmental samples. Microautoradiography provides a means of detecting individual active cells and involves incubation of cells in the presence of radiolabelled substrates, which are incorporated only by such cells. Total populations are visualised microscopically and active cells are detected by microautoradiography of material on the same slide. This is similar to combined use microautoradiography and immunofluorescence using specific antibodies, but antibodies are usually prepared from pure cultures of cultivated organisms.

Combining autoradiography with 16S rRNA probing provides greater discriminatory power, more targeted identification and detection of active cells belonging to phylogenetic groups with no known cultivated representative. This approach, termed STAR-FISH, has been used to detect the activity of marine plankton (Ouverney and Fuhrman, 1999) and cells in activated sludge Lee et al. (1999).

Links with traditional approaches

The most straightforward approach to determining the links between species diversity and ecosystem function is to isolate organisms considered to be important in ecosystem function and to assess their potential ecological impact through laboratory-based, physiological studies. Molecular studies can overcome one important limitation in this approach, by identifying which species are important. For example, rRNA-based fingerprinting techniques can determine the environmental factors that lead to changes in relative abundance of particular members of a community. These studies can now be used to inform traditional enrichment/isolation approaches, indicating which cultivated organisms represent those of relevance to ecosystem change. This prevents extensive physiological characterisation of those organisms that are selected by laboratory media and incubation conditions, but are of less importance in the environment. Molecular techniques should also aid in the discovery of conditions suitable for isolation of those organisms that are currently unculturable. Molecular ecological studies will indicate the conditions favouring their growth, informing cultivation conditions to be employed, and molecular analysis of enrichments will determine whether they are present initially and which cultivation conditions lead to their elimination or selection.

Conclusions

The application of molecular techniques has revolutionised our view of the diversity of bacteria in natural environments and is beginning to explain the forces leading to and driving changes in diversity and community structure. Early studies indicate that a similar approach can impact significantly on analysis of fungal diversity in the soil. The molecular approach eliminates a major limitation of traditional studies, the reliance on culturability, but introduces new limitations and biases, the significance of which will become apparent as studies evolve. However, comparative studies employing a range of techniques indicate that they provide a more accurate view of diversity in natural environments. They also allow more rapid identification of individual strains and of relative abundance of different groups. This, in turn, enables greater replication and a degree of quantification that provides the basis for more comprehensive application and testing of theoretical and conceptual models of microbial ecology, including links between diversity and ecosystem function. A combination of techniques is still required and traditional, cultivation-based approaches will retain an important role. Nevertheless, molecular approaches provide the potential for significant advances in our understanding of fungal diversity in natural ecosystems and offer a springboard for the application of genomics to environmental microbiology.

References

Amann R I, Krumholz L and Stahl D A 1990 Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J. Bacteriol. 172, 762–770.

Atlas R M 1984 Diversity of microbial communities. Adv. Microbial Ecol. 7, 1–47.

Borneman J and Hartin R J 2000 PCR Primers that amplify fungal rRNA genes from environmental samples. Appl. Env. Microbiol. 66, 4356–4360.

Borneman J and Triplett E W 1997 Molecular microbial diversity in soils from Eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl. Env. Microbiol. 63, 2647–2653.

Borneman J, Skroch P W, O'Sullivan K M, Palus, J A, Rumjanek N G, Jansen J L, Nienhuis J and Triplett E W 1996 Molecular microbial diversity of an agricultural soil in Wisconsin. Env. Microbiol. 62, 1935–1943.

Bruns M A, Stephen J R, Kowalchuk G A, Prosser J I and Paul E 1999 Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. Appi. Env. Microbiol. 65, 2994–3000.

Cavigelli M A, Robertson G P and Klug M J 1995 Fatty acid methyl ester (FAME) profiles as measures of soil microbial community structure. In Collins HP, Robertson G P and Klug M. J (Eds) The Significance and Regulation of Soil Biodiversity. pp 99–113 Kluwer Academic Publishers, Dordrecht, The Netherlands.

Felske A, Akkermans A D L and De Vos W M 1998 Quantification of 16S rRNAs in complex bacterial communities by multiple competitive reverse transcription PCR in temperature gradient gel electrophoresis fingerprints. Appl. Env. Microbiol. 64, 4581–4587.

Head, I M, Hiorns W D, Embley T M, McCarthy A J and Saunders J R 1993 The phylogeny of autotrophic ammonia-oxidizing bacteria as determined by analysis of 16S ribosomal RNA gene sequences. J. Gen. Microbiol. 139, 1147–1153.

Heid C A, Stevens J, Livak K J and Williams P M 1996 Real time quantitative PCR. Genome Res 6, 986–994

Jansson J K and Leser T D 1996 Quantitative PCR of environmental samples. Molecular Microbial Ecology Manual 2.7.4, 1–19.

Kowalchuk G A, Gerards S and Woldendorp J W 1997 Detection and characterization of fungal infections of *Annophila arenaria* (marram grass) roots by denaturing gradient gel electrophoresis of specifically amplified 18S rDNA. Appl. Env. Microbiol. 63, 3858–3865.

Kuske C R, Barns S M and Busch J D 1997 Diverse uncultivated bacterial groups from soils of the arid Southwestern United States that are present in many geographic regions. Appl. Env. Microbiol. 63, 3614–3621.

Lee N, Nielsen P H, Andreasen K H, Juretschko S, Nielsen J L, Schleifer K-H and Wagner M 1999 Combination of fluorescent *in situ* hybridization and microautoradiography – a new tool for structure-function analyses in microbial ecology. Appl. Env. Microbiol. 65, 1289–1297.

Liu W T, Marsh T L, Cheng H and Forney L J 1997 Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Env. Microbiol. 63, 4516–4522.

McCaig A E, Glover L A and Prosser J I 1999a Molecular analysis of eubacterial community structure and diversity in unimproved and improved upland grass pastures. Appl. Env. Microbiol. 65, 1721–1730.

McCaig A E, Phillips, C J, Stephen J R, Kowalchuk G A, Harvey S M, Herbert R A, Embley T M and Prosser J I 1999b Nitrogen cycling and community structure of β-subgroup ammonia oxidising bacteria within polluted, marine fish-farm sediments. Appl. Env. Microbiol. 65, 213–220.

McCaig A E, Glover L A and Prosser J I 2001 Numerical analysis of grassland bacterial community structure under different land management regimes using 16S rDNA sequence data and DGGE banding patterns. Appl. Env. Microbiol. 67, 4554–4559.

Muyzer G, de Waal E C and Uitterlinden A G 1993 Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Env. Microbiol. 59, 695–700.

Ovreas L and V Torsvik 1998 Microbial diversity and community structure in two different agricultural soil communities. Microbial. Ecol. 36, 303–315.

Ouverney C C and Fuhrman J A 1999 Combined microautoradiography—16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types *in situ*. Appl. Env. Microbiol. 65, 1746–1752

Phillips C J, Smith Z, Embley T M and Prosser J 11999 Phylogenetic differences between particle-associated and planktonic β-proteobacteria ammonia oxidising bacteria in the North Western Mediterranean Sea. Appl. Env. Microbiol. 65, 779–786

Purdy K J, Nedwell D B, Embley T M and Takii S 1997 Use of 16S rRNA-targeted oligonucleotide probes to investigate the occurrence and selection of sulphate-reducing bacteria in response to nutrient addition to sediment slurry microcosms from a Japanese estuary. FEMS Microbiol. Ecol. 24, 221–234.

Purkhold U, Pommerening-Roser A, Juretschko S, Schmid M C, Koops H P and Wagner M 2000 Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis. Implications for molecular diversity surveys. Appl. Env. Microbiol. 66, 5368–5382.

Radajewski S, Ineson P, Parekh N R and Murrell J C 2000 Stable-isotope probing as a tool in microbial ecology. Nature 403, 646–649.

Smit E, Leeflang P, Glandorf B, Van Elsas J D and Wernars K 1999 Analysis of fungal diversity in the wheat rhizosphere by sequencing of cloned PCR-amplified genes encoding 18S rRNA and temperature gradient gel electrophoresis. Appl. Env. Microbiol. 65, 2614–2621.

Smith Z, McCaig A E, Stephen J R, Embley T M and Prosser J I 2001 Species diversity of uncultured and cultured populations of soil and marine ammonia oxidising bacteria. Microbial. Ecol. 42, 228–237.

Stephen J R, Kowalchuk G A, Bruns M A, McCaig A E, Phillips C J, Embley, TM and Prosser J I 1998 Analysis of β-subgroup ammonia oxidiser populations in soil by DGGE analysis and hierarchical phylogenetic probing. Appl. Env. Microbiol. 64, 2958–2965.

Stephen J R, McCaig A E, Smith Z, Prosser J I and Embley T M 1996 Molecular diversity of soil and marine 16S rDNA sequences related to β-subgroup ammonia oxidising bacteria. Appl. Env. Microbiol. 62, 4147–4154.

Torsvik V, Salte K, Sorheim R and Goksoyr J 1990 Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. Appl. Env. Microbiol. 56, 776–781

Torsvik V, Sorheim R and Goksoyr J 1996 Total bacterial diversity in soil and sediment communities – a review. J. Ind. Microbiol. 17, 170–178.

Tunlid A, Hoitink H A J, Low C and White D C 1989 Characterization of bacteria that suppress *Rhizoctonia* damping-off in bark compost media by analysis of fatty acid biomarkers. Appl. Env. Microbiol. 55, 1368–1374.

Vainio E J and Hantula J 2000 Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. Mycol. Res. 104, 927–936.

Viaud M, Pasquier A and Brygoo Y 2000 Diversity of soil fungi studied by PCR-RFLP of ITS. Mycol. Res. 104, 1027–1032.