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THE USE OF 16S RDNA METHODS IN SOIL MICROBIAL ECOLOGY

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MINI-REVIEW

ABSTRACT

New and exciting molecular methods, many using the 16S small sub-unit ribosomal nucleic acid molecule, are opening the microbial "black box" in soil. These studies have added much to our knowledge of microbial diversity in soils, and are beginning to advance our understanding of the relationship between this diversity and its function in soil processes. Over the next few years, the knowledge gained from molecular studies will, we hope, lead to improvements in sustainable land management and sustainable exploitation of soil genetic resources. As we enter the third millenium, it is appropriate to review the application of 16S rDNA methods to soil microbiology. This review examines 16S ribosomal DNA (rDNA) methods and their application to soil. It mentions their limits and suggests how they may be applied in the future.

Key words: soil, bacteria, ecology, 16S rDNA

INTRODUCTION

In the preface to their book *'Brock Biology of Microoganisms'* (37) Madigan *et al.* state that:

"a new golden age of microbiology is upon us! An age in which an entire bacterial genome can be sequenced in a matter of months."

As molecular technologies facilitate explorations of gene expression I support the view that the primary task of microbial ecology is to provide an understanding of the place of microorganisms in society (5). With this in mind, when examining the impact of 16S rDNA analyses on soil bacterial ecology, one might ask to what extent our knowledge of microorganisms and their roles in natural environments has been enhanced by the introduction of molecular methods? Over the past 10 years we have seen increased interest in soil microbiology and ecology. This has been due to a better awareness that biological communities have a role in maintaining a sustainable biosphere and secondly to rapid advances in molecular biology.

The use of small sub-unit rRNAs in microbial ecology has been recently reviewed (22) and the reader is referred to this paper for a detailed review of the first 10 years of molecular microbial ecology. The first applications of nucleic acid techniques applied to molecular microbial ecology were primarily concerned with phylogenetic relationships between microorganisms determined by sequence analysis of 16S rDNA. As a result of such studies it is now widely acknowledged that the diversity of microorganisms in soils is large (3, 6, 9, 30, 32, 34, 39). The challenge for the soil microbial ecologist, as ever, is to identify the populations and guilds of microorganisms which have key functional roles in specific soil processes. Bacterial populations and guilds are now beginning to be described from similar soil niches from around the world using 16S rDNA technologies (9, 17-19, 36,46). These studies generally support previous work based on culturing organism from soils; but, importantly they indicate the active presence of novel consortia yet to be cultured. Applying molecular biological methods to investigate soils has necessitated considerable method developments. This has been because molecular biologists and biochemists have had to come to terms with contaminants such as organic matter, clay, humic acids and metals. We are now in a position to apply developed methods to test hypotheses that

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integrate microbial interactions with soil processes. This will increase our understanding of microbial community function in soils and help to clarify the importance of microbial diversity in soils. Although molecular techniques allow us to resolve changes in microbial communities at the genetic level, microbial ecologists are still faced with the age-old problems of sampling, of heterogeneity of scale and of connectivity between scales. It is in this area that small sub-unit rRNA analyses can help us resolve whether micro-niches exist independently, and if so how they interact and change. Recently developed techniques such as fluorescent in situ hybridisation (FISH) (10) offer to throw light on environments previously in the dark.

DNA EXTRACTION

Nucleic acid extraction is arguably the most important stage in a molecular microbial investigation. Consequently improved methods for the extraction of nucleic acid from different soils continue to receive attention. Molecular analysis of soil community DNA requires that the nucleic acid is first recovered from the soil. Numerous methods for DNA extraction have been described (50). Almost all of the currently used methods are variations on either a bead beating procedure in which the microbial cells are mechanically lysed in the presence of phenolic or sodium dodecylsulphate (SDS) extractants or where lysis is facilitated by heating in sodium dodecylsulphate (SDS) followed by treatment with enzymes. A problem with such analyses is how best to evaluate the efficiency of lysis and DNA recovery and how to assess whether the DNA extract obtained is representative of the indigenous microflora. Another challenge for the molecular ecologist working in soil is that the stringent protocols needed to ensure efficient lysis and quantitative nucleic acid recovery also extract large amounts of humified organic materials. Unlike clay minerals, these humic substances are soluble and contaminate the DNA inhibiting subsequent molecular analyses such as the polymerase chain reaction (PCR). For many soils these humic substances can be reliably and quickly removed using commercial kits, whilst for others more elaborate, and often labour intensive, purification procedures are required. Recently, Cullen and Hirsch (12) have described a simple and rapid procedure for the direct extraction of DNA from soils in which DNA was extracted from arable soils by bead-beating in an alkaline-SDS buffer and purified by separate PVPP (polyvinylpolypyrollidone) and Sephadex G-75 spin column chromatography. Total DNA yield was around 20µg g^{-1} dry soil which compares favourably with the values obtained from an upland soil (30 µg g^{-1} dry soil) where DNA was extracted using a lysozyme, SDS and a freeze-thaw procedure (11). In a detailed analysis of the effects of increasing bead beating times on lysis efficiency, van Elsas *et al.* (51) have shown in different soils that 90% of the cells were lysed after bead-beating for 4.5 min. Soil type had a significant impact on DNA yield and varied between 2 and 35 µg g^{-1} dry soil. These workers also showed that depending on the soil type, different soils required different purification steps to obtain amplifiable DNA.

An alternative approach to that of direct DNA extraction is to remove the microorganisms from the soil matrix prior to nucleic acid extraction. Such indirect methods rely on the efficient recovery of microorganisms from soil (25) and are now used less often. However, indirect methods offer the possibility of selectively recovering the bacterial fraction and of cleaning up the cell preparation prior to nucleic acid extraction. This can improve yields and reduce contamination by soil components.

To increase the detection of specific bacterial DNA, van Elsas *et al.* (46) have proposed a new indirect extraction protocol that by specifically desorbing bacterial cells facilitates the recovery of bacterial DNA in high yields. Although questions remain as to the efficiency and reproducibility of nucleic acid extraction protocols, advances in methodology and the rapid uptake of molecular methods by microbial ecologists have ensured that DNA extraction procedures are available for soils recovered from a wide range of environments. Recent investigations include: Canadian landfill (32), German lake mud (34), agricultural soils of the USA (3), English rhizosphere soil (36), Scottish humified soils (11), Dutch grassland soils (16), Scandanavian methane enriched soils (27), Welsh swine manure (21), Japanese paddy field soils (28) Amazon rainforest soils (4) and a wide variety of polluted soils including a torpedo fuel-spill soil (45). The continuing challenge lies in the application of these methods to increase our understanding of the links between microbial diversity and maintaining and or bioengineering soil processes.

PCR , cloning and sequencing techniques

PCR amplification of 16S rRNA genes (16S rDNA) using consensus bacterial primers and separation of the resultant PCR amplicons either by cloning, by denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) constitute the most popular molecular ecology techniques used to describe soil bacterial ecology to date (42). Clones or bands on gradient gels can be sequenced and the resultant information used to infer something about the diversity of the original sample. Over the last few years we have seen a proliferation of these studies applied to soils (14, 29, 47) as molecular techniques have been systematically applied to many diverse environments. To date, perhaps the greatest contribution these studies have made to soil microbiology is a sequence based taxonomy. Based on ribosomal sequences, the way in which we view the bacterial "Kingdom" and evolution has dramatically changed (26). Finally we have a systematic framework which also includes the uncultured soil bacteria and on which we can test hypotheses about their importance (3).

We are just beginning to expand our understanding of important soil processes, their regulation and how they might be manipulated. Thus, one of the future objectives of studying

genetic diversity in soils using 16S rDNA methods should be to facilitate the isolation of novel organisms for the biotechnology industry (53). This can be achieved and will become easier with the rapid expansion in the numbers of environmental sequences deposited on-line and the importance of such information for the design and application of oligonucleotide probes for *in situ* identification and screening of environmental bacteria (7).

Another benefit of the widespread use of 16S rDNA techniques to survey bacterial diversity in different soils, is that a number of taxa, common in geographically distinct soils, have been identified (34). Of particular interest is the phylogeography of members of the Holophaga/Acidobacterium phylum. Sequences from this group have been found in almost every analysis of 16S rDNA from soils. They have been found in Asia (41), Australia (48), North America (30), The Amazon (4), Europe (40) and Hawaii (43). In our own laboratories we have cloned sequences of this group from Antarctic soil and from the rhizosphere of *Brassica napus,* oilseed rape (canola) (30). Although the recovery of Holophaga/Acidobacterium in clone libraries does not necessarily imply an important role in soil, Ludwig *et al.* (34) have shown that they are present in bulk soil and in the rhizosphere of oil-seed rape in sufficient numbers to be probed using *in situ* hybridisation. Nonetheless, despite their ubiquitous distribution, we have as yet no understanding of the physiology of this potentially important group of organisms or of the processes they mediate. Those that have been cultured exhibit widely differing physiologies and are relatively distantly related to most of the environmental sequences. For example, *Acidobacterium capsulatum* was isolated from an acidic, mineral environment (24), whilst *Holophaga foetida* is an obligate anaerobe isolated from a black anoxic freshwater mud sample (31). *Geothrix fermentans*, the other cultured member of the group is an iron-reducing bacterium isolated from a petroleum contaminated aquifer (33). Thus, most members of this phylum have yet to be cultured, and new isolation strategies are required.

Similarly a global pattern is also emerging for an uncultured, and I predict culturable, group of bacilli. The reader's attention is drawn to the recent work of Felske *et al.* (16, 17, 19) where bacilli-like 16S rRNA sequences have dominated sequence libraries cloned from RNA extracted from acid grassland soils of the Netherlands. Likewise, Macrae *et al.* (35, 36) when comparing the bacterial diversity of rhizosphere soils with bulk soils found that the rhizosphere sequence libraries were also enriched with bacilli-like 16S rDNA sequences. It will be very interesting to see when and how this group of bacteria are cultivated and subsequently used in biotechnology.

Sequence ANALYSIS

Having written that one of the greatest contributions that 16S rDNA methods have made to soil microbiology is a sequence based taxonomy, it is relevant to note where and how ribosomal sequences can be retrieved for analysis. Sequences are generally submitted to and can be retrieved from the European Molecular Biology Laboratory (EMBL), Heidelberg, Germany; Genbank (NCBI), Bethesda, MD, USA; The DNA Database of Japan (DDBJ) Mishima, Japan, and the Ribosomal Database Project (RDP), University of Illinois, Illinois, USA. Sequences can be retrieved via the World Wide Web and new sequences compared with those held in the databases by using the basic local alignment search tool (BLAST), http://ncbi.nih.gov/cgi-bin/nph-blast?Jform=1 (1) and RDP, http://rdpwww.life.uiuc.edu/index2.html (38) (note, web addresses are frequently updated and the reader is advised to use the acronyms RDP/BLAST to guide web searches). The RDP provides a wide and excellent range of analytical services including both phenetic and phylogenetic analyses of query sequences with those on-line at the RDP. The site is free to use and for those who are not familiar with the site, a visit is strongly recommended. BLAST is likewise extremely useful for comparing query sequences with the greater number of sequences held on-line at EMBL, NCBI and DDBJ. The analysis of ribosomal sequences, including phylogenetics, warrants a review in its own right, however, for an introduction the reader is guided to the excellent text of Hillis (23).

Considerations

Over the last 2 or 3 years, as publications using 16S techniques have increased, we have seen an appreciation of the fact that in closing the rRNA loop (2) we also need to pursue culture studies if the molecular methods are to advance significantly our understanding of diversity and function in soils. This shift in attitude is in part due to recognition of the fact that like culturing and other exploratory techniques such as PLFA analysis, 16S methods have their limitations and biases. Thus, much of the recent literature also cautions against over-interpretation of data from soil community DNA arising from possible biases in PCR. Although the problems in using PCR with degenerate primers have been recognised for some time (49) only recently have they begun to be explored experimentally. Farelly *et al.* (15) using mixed cultures have highlighted the importance of gene copy number and its effect on PCR in extrapolating to relative abundance in natural environments whilst Polz *et al.* (44) using 'mutagenized templates' failed to show significant gene dosage effects. These workers have argued that the observed biases are due to PCR selection (52) with GC rich priming sites amplifying consistently better than AT rich sites. This disparity in amplification was seen as largely template inherent and additive with every cycle. However, despite this unpredictable, *a priori*, bias, the distribution of PCR amplicons were reproducible with the effects of PCR selection reduced by performing short-cycle PCR amplifications at high template concentrations.

The effects of template concentration on the PCR amplification of 16S rDNA are particularly important in many

soil studies where template DNA is often diluted to minimise the effects of humic acid contamination. In an investigation of sub-surface sediments with low microbial numbers (<10^5 g^{-1} soil), Chandler *et al.* (8) have shown that low template concentrations can result in a disproportionate representation of sequence types in the clone library (assessed using RFLP analysis). This has been attributed to very low template concentration generating random fluctuations in priming efficiency during the PCR reaction. These and similar studies suggest that we need to be especially vigilant in how we interpret molecular data derived from soils using PCR. Nonetheless, amplification of 16S rDNA and the analysis of clone libraries or amplicon diversity using techniques such as TGGE and DGGE will continue to be valuable tools for exploring microbial diversity in natural environments. What we have learned from such studies is that this must be done in parallel with other, PCR independent methods such as probing, phospholipid fatty acid analysis and culturing.

CONCLUSIONS

In reviewing the literature for this article, a search guided by the key words "soil" and "bacteria" in article titles and abstracts between January 1988 and July 1999 at the Bath Information Data Service (BIDS, telnet bids.ac.uk) returned 987 papers. Refining the search by adding "16S" returned 120 papers, which have been summarised in Table 1. In Table 1 we can see that already one in every eight cited publications working with soil and bacteria will include a 16S component and that ecological studies (organisms and environment interactions) now dominate what is published. It is important to note that significant numbers of researchers are using 16S sequences as a means to support taxonomic classifications, usually where phenetic methods have proved lengthy and inconclusive. Concurrently large numbers are continuing to develop methods aimed at linking presence in soil with function in soil processes.

Table 1. Volume of literature citing soil bacteria and 16S analyses between 1/1/98 and 1/7/99 (Data gathered from BIDS, telnet bids.ac.uk)

Study areas and number of published papers in which 16S methods have been applied to soils (1/1/98-1/7/99)				
Phylogeny	*Methods*	*Ecology*	*Taxonomy*	*Reviews*
12	24	52	28	4

In discussing developments in the use of 16S rDNA analysis over the last few years and at the potential and limitations of these techniques it is appropriate to look to the future. Although it is now accepted that microbial diversity in soils is vast, 16S rDNA techniques have yet to fulfil their potential with respect to understanding and manipulating biological function in soils. Such knowledge is essential if we are to sustain agricultural productivity, to remediate contaminated land or to cope with changes in land use concomitant with global change. To do this will require information not just on what is there (the diversity) but also the activity and size of different microbial populations and communities. If we are to manage microbial communities we must further develop methods to culture key groups of bacteria and if key groups remain unculturable, develop methods to manipulate and monitor key groups *in situ.* Some progress has already been made and there is no doubt that molecular techniques will be at the vanguard of studies designed to open the microbial 'black box' in soil. For example, the substantial work described recently by Fuchs *et al.* (20) will promote the design and optimisation of specific 16S rRNA probes for assessing the activity*, in situ,* of specific populations and guilds and should facilitate more reliable estimates of population size and community structure. Developments in quantitative PCR (30) and better protocols for RNA extraction and reverse transcriptase-PCR (13) will enable reliable quantification of active microbial populations. However, the intellectual challenge for microbial ecologists will be to use molecular methods to unravel its ecological complexity. Understanding how microbial communities are organised in soils and how they interact with their physical, chemical and biological environments remains our goal. If understood, microbial ecology can be integrated with macroecology to provide a fuller understanding of the biosphere and the place of soil microorganisms in society.

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RESUMO

Uso de métodos 16S rDNA em ecologia microbiana do solo

Novas e excitantes técnicas moleculares muitas usando a fração 16S da subunidade menor da molécula de ácido nucleico ribossomal, estão abrindo a "caixa-preta" da microbiologia do solo. Esses estudos têm acrescentado muito ao nosso conhecimento acerca da diversidade microbiana no solo, e começam a avançar nosso entendimento sobre a relação entre essa diversidade a sua função nos processos no solo. Ao longo dos próximos anos, o conhecimento obtido a partir de técnicas moleculares irão, esperamos, levar a melhoramentos do manejo de áreas sustentáveis da exploração dos recursos genéticos do solo. Com a chegada do terceiro milênio, é apropriado revermos

a aplicação das técnicas da fração 16S do rDNA em microbiologia de solo. Esta revisão examina aplicações das técnicas da fração 16S do DNA (RNA) no solo, menciona seus limites e sugere como elas poderão ser usadas no futuro.

Palavras-chave: solo, bactéria, ecologia, 16S rDNA

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