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MICROBIAL ECOLOGY- MCB 308

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DISCUSS THE VARIETY OF DIVERSE ANALYTICAL TECHNIQUES IN UNDERSTANDING THE CRITICAL ROLE OF MICROBES IN SPECIFIC ECOSYSTEMS

AQUATIC ENVIRONMENT

*Nucleic acid hybridization*

Nucleic acid hybridization using specific probes is an important qualitative and quantitative tool in molecular bacterial ecology. These hybridization techniques can be done on extracted DNA or RNA, or in situ. Oligonucleotide or polynucleotide probes designed from known sequences ranging in specificity from domain to species can be tagged with markers at the 5’-end. The sample is lysed to release all nucleic acids. Dot-blot hybridization with specific and universal oligonucleotide primers is used to quantify rRNA sequences of interest relative to total rRNA. The relative abundance may represent changes in the abundance in the population or changes in the activity and hence the amount of rRNA content. Cellular level hybridization can also be done in situ. Valuable spatial distribution information on microbial communities in natural environments can be provided by hybridization methods. One of the most popular DNA hybridization methods is FISH (Fluorescent in situ hybridization). Spatial distribution of bacterial communities in different environments such as biofilms can be determined using FISH. Lack of sensitivity of hybridization of nucleic acids extracted directly from environmental samples is the most notable limitation of nucleic acid hybridization methods. If sequences are not present in high copy number, such as those from dominant species, probability of detection is low.

*DNA Reassociation*

The kinetics of DNA reassociation reflect the variety of sequences present in the environment, thereby reflecting the diversity of the microbial community of the environment. DNA reassociation estimates diversity by measuring the genetic complexity of the microbial community. Total DNA is extracted from environmental samples, purified, denatured and allowed to reanneal. The rate of hybridization or reassociation will depend on the similarity of sequences present. As the complexity or diversity of DNA sequences increases, the rate at which DNA reassociates will decrease. The parameter controlling the reassociation reaction is concentration of DNA product (Co) and time of incubation (t), usually described as the half association value, Cot1/2 (the time needed for half of the DNA to reassociate). Under specific conditions, Cot1/2 can be used as a diversity index, as it takes into account both the amount and distribution of DNA re-association. Alternatively, the similarity between communities of two different samples can be studied by measuring the degree of similarity of DNA through hybridization kinetics.

*Restriction fragment length polymorphism (RFLP)*

Restriction fragment length polymorphism (RFLP) is another tool used to study microbial diversity. This method relies on DNA polymorphisms. In the last couple of years RFLP applications have also been applied to estimate diversity and community structure in different microbial communities. In this method, electrophoresed digests are blotted from agarose gels onto nitro-cellulose or nylon membranes and hybridized with appropriate probes prepared from cloned DNA segments of related organisms. RFLP has been found to be very useful particularly in combination with DNA-DNA hybridization and enzyme electrophoresis for the differentiation of closely related strains, and the approach seems to be useful for determination of intra species variation. RFLPs may provide a simple and powerful tool for the identification of bacterial strains at and below species level. This method is useful for detecting structural changes in microbial communities but not as a measure of diversity or for detection of specific phylogenetic groups. Banding patterns in diverse communities become too complex to analyze using RFLP since a single species could have four to six restriction fragments.

*Terminal restriction fragment length polymorphism (T-RFLP).*

Terminal restriction fragment length polymorphism (T-RFLP) is a technique that addresses some of the limitations of RFLP. This technique is an extension of the RFLP/ ARDRA analysis and provides an alternate method for rapid analysis of microbial community diversity in various environments. It follows the same principle as RFLP except that one PCR primer is labelled with a fluorescent dye, such as TET (4, 7, 2’, 7’-tetrachloro-6- carboxyfluorescein) or 6-FAM (phosphoramidite fluorochrome 5-carboxyfluorescein). PCR is performed on sample DNA using universal l6S rDNA primers, one of which is fluorescently labelled. Fluorescently labelled terminal restriction fragment length polymorphism (FLT-RFLP) patterns can then be created by digestion of labelled amplicons using restriction enzymes. Fragments are then separated by gel electrophoresis using an automated sequence analyzer. Each unique fragment length can be counted as an Operational Taxonomic Unit (OTU), and the frequency of each OTU can be calculated. The banding pattern can be used to measure species richness and evenness as well as similarities between samples. TRFLP, like any PCR-based method, may underestimate true diversity because only numerically dominant species are detected due to the large quantity of available template DNA. Incomplete digestion by restriction enzymes could also lead to an overestimation of diversity. Despite these limitations, some researchers are of the opinion that once standardized, T-RFLP can be a useful tool to study microbial diversity in the environment, while others feel that it is inadequate. T-RFLP is limited not only by DNA extraction and PCR biases, but also by the choice of universal primers. None of the presently available universal primers can amplify all sequences from eukaryote, bacterial and archaeal domains. Additionally, these primers are based on existing 16S rRNA, 18S rRNA or Internal Transcribed Spacer (ITS) databases, which until recently contained mainly sequences from culturable microorganisms, and therefore may not be representative of the true microbial diversity in a sample. In addition, different enzymes will produce different community fingerprints. T-RFLP has also been thought to be an excellent tool to compare the relationship between different samples. T-RFLP has been used to measure spatial and temporal changes in bacterial communities, to study complex bacterial communities, to detect and monitor populations and to assess the diversity of arbuscular mycorrhizal fungi (AMF) in the rhizosphere of *Viola calaminaria* in a metal-contaminated soil.

*Ribosomal intergenic spacer analysis (RISA)/ Automated ribosomal intergenic spacer analysis (ARISA) /Amplified ribosomal DNA restriction analysis (ARDRA)*

Similar in principle to RFLP and T-RFLP, RISA, ARISA and ARDRA provide ribosomal-based fingerprinting of the microbial community. In RISA and ARISA, the intergenic spacer (IGS) region between the 16S and 23S ribosomal subunits is amplified by PCR, denatured and separated on a polyacrylamide gel under denaturing conditions. This region may encode tRNAs and is useful for differentiating between bacterial strains and closely related species because of heterogeneity of the IGS length and sequence. Sequence polymorphisms are detected by silver staining in RISA. In ARISA, fluorescently labelled forward primer is detected automatically. Both RISA and ARISA method can deduce highly reproducible bacterial community profiles. Limitations of RISA include requirement of large quantities of DNA, relatively longer time requirement, insensitivity of silver staining in some cases and low resolution. ARISA has increased sensitivity than RISA and is less time consuming but traditional limitations of PCR also applies for ARISA. RISA has been used to compare microbial diversity in soil, in the rhizosphere of plants, in contaminated soil and in response to inoculation.

*DNA microarrays*

More recently, DNA–DNA hybridization has been used together with DNA microarrays to detect and identify bacterial species or to assess microbial diversity. This tool could be valuable in bacterial diversity studies since a single array can contain thousands of DNA sequences with high specificity. Specific target genes coding for enzymes such as nitrogenase, nitrate reductase, naphthalene dioxygenase etc. can be used in microarray to elucidate functional diversity information of a community. Sample of environmental ‘standards’ (DNA fragments with less than 70% hybridization) representing different species likely to be found in any environment can also be used in microarray. Another DNA microarray based technique for analyzing microbial community is Reverse Sample Genome Probing (RSGP). This method uses genome microarrays to analyze microbial community composition of the most dominant culturable species in an environment. RSGP has four steps: (1) isolation of genomic DNA from pure cultures; (2) cross-hybridization testing to obtain DNA fragments with less than 70% crosshybridization. (DNA fragments with greater than 70% cross-hybridization are considered to be of the same species). (3) Preparation of genome arrays onto a solid support and (4) random labelling of a defined mixture of total community DNA and internal standard. This method has been used to analyze microbial communities in oil fields and in contaminated soils. Like DNA–DNA hybridization, RSGP and microarrays have the advantages that these are not confounded by PCR biases. Microarrays can contain thousands of target gene sequences but it only detects the most abundant species. In general, the species need to be cultured, but in principle cloned DNA fragments of unculturables could also be used. The diversity has to be minimal or enriched cultures should be used for this method. Otherwise, cross-hybridization can become problematic. Using genes or DNA fragments instead of genomes on the microarray offers the advantages of eliminating the need to keep cultures of live organisms, as genes can be cloned into plasmids or PCR can continuously be used to amplify the DNA fragments. In addition, fragments would increase the specificity of hybridization over the use of genomes and functional genes in the community could be assessed.

 *Denaturant gradient gel electrophoresis (DGGE)/Temperature gradient gel electrophoresis (TGGE)*

In denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), DNA fragments of same length but with different base-pair sequences can be separated. DNA is extracted from natural samples and amplified using PCR with universal primers targeting part of the 16S or 18S rRNA sequences. The separation is based on the difference in mobility of partially melted DNA molecules in acrylamide gels containing a linear gradient of DNA denaturants (urea and formamide). Sequence variation within the DNA fragments causes a difference in melting behaviour, and hence in separation in denaturing gradient gels. The melting of the products occurs in different melting domains, which are stretches of nucleotides with identical melting temperatures. Analyzing Diversity of Microbial Communities 27 Sequence variations in different fragments will therefore terminate migration at different positions in the gel according to the concentration of the denaturant. Theoretically, DNA sequences having a difference in only one base-pair can be separated by DGGE. TGGE employs the same principle as DGGE but in this method the gradient is temperature rather than chemical denaturants. Advantages of DGGE/TGGE include reliability, reproducibility, rapidness and low expense. As multiple samples can be analyzed simultaneously, tracking changes in microbial population in response to any stimuli or adversity is possible by DGGE/TGGE. Limitations of DGGE/ TGGE include PCR biases, laborious sample handling, and variable DNA extraction efficiency. It is estimated that DGGE can only detect 1–2% of the microbial population representing dominant species present in an environmental sample. In addition, DNA fragments of different sequences may have similar mobility characteristics in the polyacrylamide gel. Therefore, one band may not necessarily represent one species and one bacterial species may also give rise to multiple bands because of multiple 16S rRNA genes with slightly different sequences. DGGE profiles have successfully been used to determine the genetic diversity of microbial communities inhabiting different temperature regions in a microbial mat community, and to study the distribution of sulphate reducing bacteria in a stratified water column.

*Metagenomic analysis of microbial communities*

Metagenomics is defined as the functional and sequence-based analysis of the collective microbial genomes that are contained in an environmental sample. In metagenomics, the collective genome (metagenome or microbiome) of coexisting microbes – called microbial communities is randomly sampled from the environment and subsequently sequenced. By directly accessing the collective genome of cooccurring microbes, metagenomics has the potential to give a comprehensive view of the genetic diversity, species composition, evolution, and interactions with the environment of natural microbial communities. Community genomic datasets can also enable subsequent gene expression and proteomic studies to determine how resources are invested and functions are distributed among community members. Ultimately, genomics can reveal how individual species and strains contribute to the net activity of the community.

SOIL ENVIRONMENT

*Sole-carbon-source Utilization (SCSU)*

The Sole-Carbon-Source Utilization (SCSU) [also known as Community Level Physiological Profiling (CLPP)] system was introduced by Garland and Mills (1991). This was initially developed as a tool for identifying pure cultures of bacteria to the species level, based upon a broad survey of their metabolic properties. SCSU examines the functional capabilities of the microbial population, and the resulting data can be analyzed using multivariate techniques to compare metabolic capabilities of communities.

*Phospholipid fatty acid (PLFA) analysis*

The fatty acid composition of microorganisms has been used extensively to aid microbial characterization. Taxonomically, fatty acids in the range C2 to C24 have provided the greatest information and are present across a diverse range of microorganisms. The fatty acid composition is stable, and is independent of plasmids, mutations or damaged cells. The method is quantitative, cheap, robust and with high reproducibility. However, it is important to notice that the bacterial growth conditions are reflected in the fatty acid pattern. This method is also known as the fatty acid methyl ester (FAME) analysis.

Although FAME analysis is used to study microbial diversity, this fatty acid analysis method might fraught with limitations, when total organisms are used. This may obscure detection of minor species in the population.