

MICROBIAL COMMUNITIES AND THEIR INTERACTIONS IN SOIL AND RHIZOSPHERE ECOSYSTEMS

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■ **Abstract** Since the first estimate of prokaryotic abundance in soil was published, researchers have attempted to assess the abundance and distribution of species and relate this information on community structure to ecosystem function. Culture-based methods were found to be inadequate to the task, and as a consequence a number of culture-independent approaches have been applied to the study of microbial diversity in soil. Applications of various culture-independent methods to descriptions of soil and rhizosphere microbial communities are reviewed. Culture-independent analyses have been used to catalog the species present in various environmental samples and also to assess the impact of human activity and interactions with plants or other microbes on natural microbial communities. Recent work has investigated the linkage of specific organisms to ecosystem function. Prospects for increased understanding of the ecological significance of particular populations through the use of genomics and microarrays are discussed.

CONTENTS

INTRODUCTION TO THE PROBLEM	212
CULTURE-INDEPENDENT ASSESSMENTS OF MICROBIAL COMMUNITIES	213
Early Analyses of Microbial Diversity in Soils by Molecular Means	213
CULTURE-INDEPENDENT METHODS OF ASSESSING MICROBIAL DIVERSITY	214
PCR-Based Methods	214
Alternatives to PCR Approaches	216
Methods to Assess Community Function in Soil	217
CHANGES IN UNCULTURED BACTERIAL COMMUNITIES WITH DISTURBANCE	218
Heavy Metals	218

Addition of Pollutants to Soil	219
Pesticide Treatment	219
Agricultural Management	220
MORE DIRECT COMPARISONS BETWEEN	
CULTURE-DEPENDENT AND CULTURE-INDEPENDENT	
ASSAYS OF SOIL MICROBIAL DIVERSITY	221
SOIL PARTICLE SIZE	222
MICROBIAL DIVERSITY OF THE SOIL-ROOT	
INTERFACE: THE RHIZOSPHERE	222
Influence of the Host Plant on Rhizosphere	
Bacterial Communities	222
Transgenic Plants and Microbial Diversity	223
Bacterial Rhizosphere Communities and Plant Disease	224
Bacterial Antibiotic Production and Rhizosphere	
Microbial Diversity	224
Rhizosphere Microbial Diversity and Plant Nutrient Status	225
Epiphytic Bacterial Diversity	225
NONMETHANOGENIC ARCHAEA IN SOIL	225
FUTURE NEEDS IN UNDERSTANDING	
MICROBIAL DIVERSITY	226

INTRODUCTION TO THE PROBLEM

From the classic paper of Torsvik et al. (128) came the first culture-independent estimate of the number of prokaryotic genomes in soil. That estimate of 4600 distinct genomes per gram of soil was determined by the reassociation time of total community DNA compared with a standard curve of reassociation kinetics of a known number of cultured genomes. The classical ecological approach for the description of an ecosystem is to first characterize the community structure by identification and enumeration of the species present, and then to assign roles in ecosystem function to species or groups. This strategy, typically employed by ecosystem and population ecologists, has not always been practical for microbial ecologists.

Traditionally, taxonomic classification of bacteria has been determined based on metabolic, morphologic, and physiological traits (42, 55). This approach emulates the methodological approach of botanists and zoologists; however, it requires the isolation and cultivation of individual bacterial species. Assessments of bacterial communities from a number of environments have found that the fraction of cells that may be cultured is not representative of the abundance or diversity of the microbial community present in the environment; it is often observed that direct microscopic counts exceed viable cell counts by several orders of magnitude [reviewed in (4, 54, 94)]. Clearly, culture-based methodology is inadequate to serve the needs of microbial ecologists seeking to describe the diversity of bacterial communities in environmental samples.

CULTURE-INDEPENDENT ASSESSMENTS OF MICROBIAL COMMUNITIES

The rRNA molecules have long been recognized for their utility as molecular chronometers (136). These molecules occur in all organisms and possess a high degree of structural and functional conservation. The larger rRNA molecules contain many domains with independent rates of sequence change (related to their structural and functional conservation). Examination of these changes over time allows phylogenetic relationships to be measured. A number of methods have been developed that exploit this sequence divergence among taxa to examine microbial community structure. These culture-independent methods for microbial community analysis most often utilize polymerase chain reactions (PCR) to amplify phylogenetic markers from DNA extracted from the microbial community. Methodologies commonly used for microbial community analyses are summarized below. For more detailed information on these methods and their limitations, the reader is referred to recent reviews (46, 76, 103, 127, 130).

Early Analyses of Microbial Diversity in Soils by Molecular Means

In the first assessments of soils by culture-independent means, *Proteobacteria* were found to dominate 16S rDNA clone libraries using template DNA from a Queensland soil (68, 124). A more diverse population was found in a Japanese soybean field (129). However, in these papers, few clones were sequenced. In a larger study, three bacterial divisions, the *Proteobacteria*, the *Fibrobacter*, and the low G + C gram-positive bacteria, were represented in nearly 60% of the 16S rDNA clones with a Wisconsin pasture soil as the source of the template DNA (14). In sharp contrast to these agricultural soils, analysis of 16S rDNA clones from a Siberian tundra soil showed that over 60% of the clones belonged to the *Proteobacteria* and 16% to the *Fibrobacter* (144).

The publication of this early work encouraged analyses of soil microbial diversity under a wide variety of conditions, which are summarized below. These comparative studies must still be considered preliminary, as no known methods are capable of efficiently assessing the fate of over 10,000 distinct organisms per gram of soil over time and space. We have learned what divisions of bacteria commonly dominate soil, but we do not know their ecological significance. When changes are observed with soil treatments, we still do not know whether these changes affect ecosystem function. The current era of investigation can be viewed as the descriptive phase, which is necessary prior to a testing phase where we will learn the role and perhaps the functional redundancy of the perhaps hundreds of millions of operational taxonomic units in soils on earth.

Given the breadth of the current literature it is difficult for any review of this length to be comprehensive. For those important works that we have neglected to

mention, we must apologize in advance. The reader is also referred to a number of important recent reviews by Amann (2), DeLong & Pace (29), Hughes et al. (56), Johnsen et al. (60), Øvreås (90), and Pace (92). Each of these covers topics that are now mentioned here and in some cases offers more detail on particular issues.

Prior to continuing this discussion of microbial diversity in soils, a description of the methods used for such assessments is necessary.

CULTURE-INDEPENDENT METHODS OF ASSESSING MICROBIAL DIVERSITY

PCR-Based Methods

Community analyses based on PCR have a number of steps that may introduce biases, starting with DNA extraction. Bacterial cell structure varies among taxonomic groups, with some bacteria being more easily disrupted than others. In addition, environmental factors require special consideration for both sample collection and DNA extraction. Inhibition of PCR by environmental compounds has been reviewed by Wilson et al. (135). Methods for sample collection and DNA extraction must take into account such factors as coextraction of humic substances from soil and low bacterial cell density in some environments, and at the same time optimize lysis of structurally different cells. Niemi et al. (86) demonstrated that soil bacterial community profiles differed depending on the DNA extraction and purification method utilized. Methods that include mechanical lysis using a bead beater were found to yield the most consistent results.

Despite these caveats, PCR-based community analysis methods are commonly used because of the ease with which many samples can be analyzed and the ability to tailor the analysis to examine particular organisms or taxa of interest through the use of universal or group-specific primers (19, 47, 66, 107). A number of community “fingerprint” methods are commonly used to assess differences in community composition between samples or treatments or to assess changes in microbial communities over time. Such techniques as ribosomal intergenic spacer analysis (RISA) (15, 100, 101, 107), denaturing gradient gel electrophoresis (DGGE) (83), temperature gradient gel electrophoresis (TGGE) (49), single-strand-conformation polymorphism (SSCP) (115, 116), ITS-restriction fragment length polymorphism (ITS-RFLP) (27), random amplified polymorphic DNA (RAPD) (44, 142), or amplified ribosomal DNA restriction analysis (ARDRA) (78) yield complex community profiles that do not directly offer phylogenetic information but do allow analysis and comparisons of community composition. Differences in electrophoretic profiles between samples reflect differences in community composition and abundance of individual microbial populations in a community. Although the fingerprint obtained from an environmental sample cannot reveal the taxonomic composition of a microbial community, phylogenetic information about particular community members may be obtained by isolation and sequence analysis of bands of interest.

A number of approaches have been developed to improve the detection and resolution of fragment analysis, including automated ribosomal intergenic spacer analysis (ARISA) (39, 102), length heterogeneity PCR (LH-PCR) (106, 126), and terminal restriction fragment length polymorphism (T-RFLP) (65, 70, 77, 89). For details of these methods, the reader is referred to the original literature. These methods utilize a fluorescently labeled oligonucleotide primer for PCR amplification and an automated system such as the Applied Biosystems capillary or gel electrophoresis instruments for separation and detection of PCR fragments. Automation of the procedure increases sample throughput and allows the rapid analysis of bacterial community structure. The high resolution offered by automated electrophoresis instruments and the high sensitivity of fluorescence detection increase the number of peaks detected compared to methods that use standard gel electrophoresis and detection. In addition, the band intensity can be measured more precisely by fluorescence detection methods, which allows a more accurate comparison of community profiles. Ranjard et al. (102) point out that this level of sensitivity may have undesirable aspects, as it may introduce variability in community profiles that has no biological origin. When the fluorescent fragment analysis techniques are used, information on the relative abundance of individual fragments (presumed to represent different bacterial taxa) is collected. These data are analogous to typical ecological data about species composition and abundance, and as such can be used to express the diversity of a community using indices of ecological diversity such as the Shannon-Weaver diversity index, Sorenson's similarity index, or the Bray-Curtis similarity index, as well as measures such as richness or evenness (73).

Of the fragment analysis methods listed above, only T-RFLP offers phylogenetic information directly without further sequencing of the fragments. Fragment length obtained from T-RFLP analysis of a microbial community may be compared to the expected terminal restriction fragment length obtained from analysis of known 16S rRNA gene sequences (77). In practice, however, the complexity of T-RFLP profiles obtained from environmental samples can hinder phylogenetic assignment of individual fragments (65, 70, 76). When more specific phylogenetic information is desired, researchers employ the more laborious strategy of constructing a clone library from the amplified phylogenetic markers (6, 16, 30, 51, 131). This approach exchanges rapid analysis of microbial community composition for fine-scale taxonomic assignment of dominant community members. The logistics of sequencing sufficient and statistically significant numbers of clones to describe the diversity of an environment make this a cumbersome technique for the comparison of microbial communities. Clone libraries are most useful for identification and characterization of previously undescribed species or for augmenting molecular fingerprinting techniques (65, 80). Approaches utilizing DNA microarrays are being developed to increase the application of clone libraries for comparisons of microbial communities (121).

While correlations between the distribution of PCR-amplified phylogenetic markers and species distribution have limitations owing to the presence of multiple

rRNA operons in bacteria (35, 98) and PCR and cloning biases (23, 97, 105, 126), molecular methods for community analysis can reveal the presence of microorganisms that remain intractable to traditional cultivation techniques. Fernández et al. (37) found that the effects of these biases are minimized when relative changes are studied in the same ecosystem and when replicate community profiles are produced.

The majority of studies that utilize these PCR-based techniques are carrying out community analyses using the ribosomal RNA operon, typically the 16S rRNA gene, though the 5S rRNA gene is sometimes used (53). Marsh (76) summarized several bacterial housekeeping genes with potential as phylogenetic markers. These include genes for heat shock proteins, glutamine synthetase, ATPases, and topoisomerases (76). When specific traits or functional characteristics are under investigation, phylogenetic markers other than the rRNA genes can be used to characterize microbial communities. For soil microorganisms, functions associated with nitrogen metabolism have been widely used for community analysis. The phylogenetic markers used in these studies include a structural gene for nitrogenase (*nifH*) [studies reviewed by (81, 95)], nitrous oxide reductase (*nosZ*) (111, 112), and nitrite reductase genes (*nirK* and *nirS*) (18). Genes involved in methane oxidation (*pmoA*, *mmoB*, and *mxoF*) have also been used to characterize soil microbial communities (47). When a particular function is restricted to specific bacterial taxa, 16S rRNA sequence may be used to differentiate these community members. This approach is used to study autotrophic ammonia oxidizers as well as methane-oxidizing bacteria; PCR primers specific for the 16S rDNA of the closely related organisms capable of these functions can be used to carry out the community analyses described above (19, 47).

Alternatives to PCR Approaches

Methods that examine physiological or metabolic characteristics of microbial communities are alternatives to PCR-based approaches. Fatty acid methyl ester (FAME) profiles and phospholipid fatty acid analysis have been used extensively to characterize the composition of soil microbial communities [(57, 106) and references therein].

Direct microscopic examinations are also important for analysis of microbial communities. Fluorescence in situ hybridization (FISH) can be used to evaluate the distribution and function of microorganisms in situ (3, 5). This method uses oligonucleotide hybridization probes complementary to regions of the 16S rRNA gene for determination of in situ abundance. Like the PCR-based methods, this technique can be customized to target specific groups of organisms. To place the dynamics of important populations within the context of community-level phenomena, FISH can be used in combination with DAPI (4',6'-diamidino-2-phenylindole), 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride (INT)-formazan, or 5-cyano-2,3-ditoly tetrazolium chloride (CTC) staining (64, 96) to determine the contribution made by the populations of interest to total

abundance or active cell count. However, the low throughput of FISH limits its application for comparison of large numbers of samples.

Careful morphological analysis of bacterial cells can provide powerful information on the diversity, microbial abundance, and two-dimensional spatial distribution of microbial community members. A computer-aided system has been developed by the Center for Microbial Ecology at Michigan State University to assist in such assessments. CMEIAS (Center for Microbial Ecology Image Analysis System) is a semi-automated analysis tool that uses digital-image processing and pattern-recognition techniques in conjunction with microscopy to gather size and shape measurements of digital images of microorganisms to classify them into their appropriate morphotype, allowing culture-independent quantitative analysis of the diversity and distribution of complex microbial communities (69). This tool holds much promise for automating a tedious but important evaluation of microbial communities.

Methods to Assess Community Function in Soil

As microbial ecology involves the study of both the structure and function of an ecosystem, meaningful assessments of microbial communities must consider not only the abundance and distribution of species but also the functional diversity and redundancy present in a microbial community. Gaston (41) has described functional diversity as the number of distinct processes (functions) that can potentially be performed by a community, whereas functional redundancy is measured as the number of different species within the functional groups present in a community. The diversity of metabolic functions possessed by microbial communities is often examined using BIOLOG GN substrate utilization assays (40, 50, 59, 122), which assess the ability of the community as a whole to utilize select carbon substrates. This method has the inherent biases of other culture-based approaches; however, the resulting metabolic fingerprint may not be an accurate representation of the functional diversity of the natural microbial community (91, 122). An alternative technique that avoids misrepresentation of functional diversity due to culture bias assesses community response (measured as CO₂ respiration) after the addition of selected carbon substrates directly to the soil environment (28).

To gain better insight to the microbial processes within an ecosystem, it is essential to study functional diversity in combination with taxonomic diversity. Recent studies have attempted to characterize the portion of the microbial community that responds to nutrient availability by comparing community fingerprints after incubation in individual BIOLOG wells (122) or by isolating DNA from microbial community populations that responded to nutrient addition by uptake and incorporation of a thymidine nucleotide analog, bromodeoxyuridine (BrdU) (13). Molecular fingerprint analysis of the responsive portion of the microbial community (as defined by BrdU labeling) was also used to assess the functional redundancy of bacterial communities along a vegetation gradient (143).

A DNA microarray technique for the simultaneous identification of ecological function and phylogenetic affiliation of microbial populations has been recently developed (11). The approach combines a community-specific 16S rDNA-based oligonucleotide array (functional diversity array) with incubations of the microbial community with various radiotracer substrates. Total RNA extracted from the in situ incubations is hybridized to the microarray of species-specific probes, which allows the identification of populations that were active in the metabolism of the labeled substrates. This approach permits the assessment of growth rate and substrate utilization of individual microbial populations within a community. In addition, whole-genome DNA microarrays specific for a single organism can be used to analyze related organisms and may ultimately prove useful for community analysis (1, 31, 82). These arrays can be used for both gene discovery as well as for analysis of gene expression in the environment.

CHANGES IN UNCULTURED BACTERIAL COMMUNITIES WITH DISTURBANCE

The methods available to assess the effects of pesticides on bacterial diversity in soils were recently reviewed by Johnsen et al. (60). That discussion is not repeated here. Rather, we describe the results to date of the efforts made to assess how agricultural management, pesticides, and pollutants have altered the microbial landscape.

Heavy Metals

A soil treated with sludge containing either high or low amounts of heavy metals was analyzed for soil bacterial diversity of three subdivisions of the Proteobacteria: the Cytophaga-Flavobacterium division, the gram-positive high G + C division, and the gram-positive low G + C division. All were measured using a dot blot hybridization procedure (109). Heavy metal treatment had the greatest influence on two of these taxa. Sandaa et al. (109) found that the abundance of the α -Proteobacteria more than doubled while the Cytophaga-Flavobacterium division abundance declined by more than two thirds with the high heavy metal treatment. Other taxa seemed to decline with high heavy metal treatment, but their low abundance with the low heavy metal treatment made a quantitative assessment of the decline difficult. In another study, Sandaa et al. (110) showed that the number of prokaryotic genomes per gram of wet weight of soil declined eightfold following many years of heavy metal treatment. With the exception of the α -Proteobacteria, all phylogenetic taxa examined declined as a percentage of the total number of prokaryotes in the soil. The percentage of α -Proteobacteria more than doubled with the heavy metal treatment.

Addition of Hg(II) to a silt loam caused an increase in abundance of two RISA bands (100). These bands were excised, sequenced, and identified as having originated from a *Clostridium*-like gram-positive organisms and a *Ralstonia*-like

β -Proteobacterium. Verification of the identity of these bacteria was done by hybridization. This is an excellent example of the identification of uncultured bacteria following a given treatment.

Addition of Pollutants to Soil

During a pentachlorophenol enrichment in a reactor containing a soil slurry, the dominant organisms found after a period of enrichment were related to the genus *Sphingomonas* (8). Incubation of a soil sample with methane resulted in the enrichment of a group of putative methylotrophic α -Proteobacteria distantly related to known methylotrophs as well as an increase in type II methanotrophs (59a). Øvreås & Torsvik (91) found that methane treatment reduced overall bacterial diversity as measured by DGGE while enriching methanotrophs.

In assessments of bacterial diversity in soil microcosms using TGGE, bacterial diversity declined following treatment with chlorinated benzoates compared to the untreated control (99). *Burkholderia*-like organisms were found to increase with the addition of chlorinated benzoates in these experiments.

The DGGE profile of 16S rDNA from a polyaromatic hydrocarbon-contaminated sandy loam was considerably less diverse than those of noncontaminated soils (84). However, a direct assessment of the effects of polyaromatic hydrocarbons cannot be made since there was no uncontaminated sample available from the same site.

Few pollutants in soils have been examined for their effects on soil microflora from a culture-independent perspective. As the techniques to make such assessments continue to improve and simplify, such analyses may become a routine part of environmental assessments required by governmental agencies prior to the use of a new compound in the environment. However, with such assessments must come ideas on how such results can be properly interpreted for risk assessment analysis. Does an impact on microbial diversity have a significant effect on soil function? Does the functional redundancy of microbial processes render microbial diversity analyses based on phylogenetics meaningless with regard to ecosystem function? If that is the case in some circumstances, should microbial processes be measured along with the taxonomic diversity assays?

Pesticide Treatment

El Fantroussi et al. (34) examined the effect of three phenyl urea herbicides on microbial communities in soils over an 11-year period. All three herbicides significantly decreased the number of culturable heterotrophic bacteria. BIOLOG GN fingerprint analysis also showed that the treated communities differed significantly compared to the control. A striking result of this work is the apparent decline of uncultured *Acidobacterium* upon treatment with any of the three herbicides. Uncultured *Acidobacterium* are commonly found in culture-independent analyses of soils. It is not clear whether the decline is caused directly by the herbicides or as a consequence of the changes in the macroflora community resulting from herbicide use. Treatment of soil with the fungicide triadimefon caused a decline in organic

carbon and soil microbial biomass but no decline in microbial DNA diversity as measured with RAPD random primer amplification (142). This can be explained by the common contradiction that although fungi can comprise a large proportion of soil biomass, fungal DNA concentrations in soil are low (45).

Xia et al. (138) evaluated microbial community response to the experimental application of 2,4-dichlorophenoxyacetic acid (2,4-D) using RAPD fingerprints. No changes in community structure were observed in response to 2,4-D application to three different soils. Hybridization studies indicated that application of 2,4-D at the recommended application rates did not select for bacterial populations capable of 2,4-D degradation.

Two culture-dependent studies on the effects of herbicides on soil bacterial diversity present conflicting results. Nicholson & Hirsch (85) showed an increase in culturable bacterial populations in soils treated with herbicides such as glyphosate. The authors thought that the increased crop yield resulting from the herbicide treatment might have contributed to higher bacterial numbers. In contrast, Busse et al. (22) found lower bacterial numbers in a pine plantation treated with glyphosate compared to the untreated control. Culture-independent analyses are needed to resolve this question.

Fumigants are used widely in high-value crops for the control of eukaryotic soil-borne pests such as fungal pathogens, nematodes, and weeds. Ibekwe et al. (58) studied their effect on soil prokaryotic communities from a culture-independent perspective. Of four fumigants used, methyl bromide caused the greatest and longest-lasting impact on soil bacterial diversity. Chloropicrin had virtually no impact.

The few studies published to date from a culture-independent perspective suggest that pesticides have little impact on soil bacterial diversity. However, so little has been done in so few locations with so few pesticides, that no conclusions can be drawn at this time. This is an area that is ripe for more investigation.

Agricultural Management

Through the use of ribosomal intergenic space analysis (RISA), deforestation in Amazonia was shown to have a profound, qualitative impact on soil bacterial diversity (15). This early work on a culture-independent assessment of the effects of land management on microbial diversity has since been followed by several more quantitative measurements of the effects of land use. Another analysis of community changes in tropical soils with deforestation was done by Nüsslein & Tiedje (88). The G + C content of the pasture soil DNA was significantly higher than that of the forest soil DNA. Whereas the Fibrobacter were dominant in the forest soil, the β - and α -Proteobacteria dominated the pasture soil.

Improved and unimproved Scottish grasslands differing in fertilizer regimes and plant cover were assessed for microbial diversity using 16S rDNA clone libraries (79). Both pastures were dominated by α -Proteobacteria (about 40% of the total clones) followed by the actinomycetes (13.3% of the total). Indices of

diversity including the Shannon-Weaver index as well as evenness and dominance measurements were similar between the two pastures.

Grasslands in the Netherlands taken out of agricultural production over a period of 30 years were examined for changes in microbial diversity (36). The multiple competitive RT-PCR procedure used did not have sufficient resolution to distinguish those pastures currently in agricultural production from those taken out of production 30 years earlier. Similarly, the application of sewage sludge to a grassland site for over 100 years failed to confer a measurable change in soil microbial diversity as determined by fatty acid methyl ester patterns and carbon substrate utilization by the community (67).

Through a culture-independent analysis of soils collected from the Kellogg Biological Station's Long Term Ecological Research project of Michigan State University, Buckley & Schmidt (21) found that the microbial diversity of cultivated fields differed little from each other regardless of the specific agricultural management regime. However, the bacterial diversity of the managed soils were significantly different from soils of nearby fields that had never been cultivated. This analysis was done using 16S rDNA taxa-specific probes and T-RFLP analysis of amplified 16S rDNA. This is an excellent site for such analyses because the Long Term Ecological Research sites have long-term data on the temporal and spatial variability of a wide range of physical, chemical, and biological properties of the experimental location. These long-term data allow the investigator to correlate changes in microbial communities with ecosystem processes. An interesting follow-up to the work of Buckley & Schmidt (21) would be to address the question of the temporal variability of bacterial diversity in these soils and determine whether any of the observed variability is correlated to any of the physical and chemical characteristics of these soils.

MORE DIRECT COMPARISONS BETWEEN CULTURE-DEPENDENT AND CULTURE-INDEPENDENT ASSAYS OF SOIL MICROBIAL DIVERSITY

The microbial communities of four arid soils from northern Arizona were compared by identifying cultured isolates and by restriction fragment-length polymorphism and sequence analysis of 16S rDNA clones derived from soil DNA (32). Seven bacterial divisions were represented among the clone libraries while only three were found among the isolates. *Acidobacterium*-related organisms comprised nearly half the organisms identified in the clone libraries, while nearly 80% of the isolates were gram-positive strains. As expected, the culture bias failed to identify most of the organisms observed in the clone libraries.

Similar results were obtained in a wheat field from Holland where *Acidobacterium* and the Proteobacteria dominated the uncultured organisms and gram-positive organisms dominated the culture collection (123). In addition, Smit et al. (123) looked at seasonal changes in these populations and found that samples

taken in July were significantly different than those taken during other times of the year.

Analysis of cultured isolates from a sandy loam and an organic soil from Norway suggested that the bacterial diversity in the two soils was similar (91). However, as the DGGE profiles were not digitized, the authors were unable to assess differences in diversity by culture-independent means. Nevertheless, one of the hallmark assays from the Torsvik group was performed. Using thermal denaturation and reassociation of community DNA, the authors showed that the organic soil possessed 10–62-fold higher genome complexity than the sandy loam soil. This result confirms the need to assess diversity by culture-independent means.

SOIL PARTICLE SIZE

Ranjard et al. (101) used RISA to show that microbial diversity varies with soil particle size. Although it is not surprising that different organisms can occupy niches of different size, this is the first paper to demonstrate this from a culture-independent perspective. Sessitsch et al. (117) took these ideas one step further and showed that microbial diversity, as determined by T-RFLP profiles, increases with decreasing particle size. Larger particles were dominated by the α -Proteobacteria while the *Holophaga/Acidobacterium* were most common in clay particles.

MICROBIAL DIVERSITY OF THE SOIL-ROOT INTERFACE: THE RHIZOSPHERE

The rhizosphere is defined as the soil surrounding the roots that is influenced by living roots. This influence may occur by root exudation of carbon substrates that affect microbial communities. Shortly after the reports of culture-independent analyses of bulk soil were published, many investigators around the world turned their attention to the rhizosphere where so many interactions between microorganisms and plants take place. The number of issues that can be studied is limited only by the imagination. We review a set of these below.

Influence of the Host Plant on Rhizosphere Bacterial Communities

Smalla et al. (122a) used DGGE to distinguish microbial communities in bulk soils versus those in the rhizospheres of strawberry, canola, and potato. Rhizosphere communities differed significantly from bulk soil communities. Canola and potato rhizosphere communities were more similar to each other than they were to strawberry. Sequencing of some of the DGGE bands excised from rhizosphere sample gels revealed that most were derived from gram-positive strains. Plant species, root zone, and soil type all influence the rhizosphere bacterial community in the DGGE analysis of 16S rDNA by Marschner et al. (75). However, no data

were provided concerning the identity of the specific organisms affected by these treatments. Similarly, the presence of rye or alfalfa roots in the soil influenced the rhizosphere community more strongly than did soil type. Kaiser et al. (63) found that the rhizosphere of canola was dominated by the α -Proteobacteria subdivision and the Cytophaga-Flavobacterium-Bacteroides division. This was in contrast to the cultured isolates from the rhizosphere that were dominated by organisms from the β - and γ -Proteobacteria subdivisions.

Normander & Prosser (87) assessed barley phytosphere bacterial diversity by using DGGE profiles. They found that plant age up to 36 days had little influence on the rhizosphere communities. The rhizosphere community was more similar to the bulk soil community than it was to the endophytic community. This suggests that rhizosphere bacteria are of soil origin, whereas many of the endophytic bacteria are seed borne.

Marilley & Aragno (74) sequenced 16S rDNA clones prepared from DNA templates collected from bulk soil, the rhizosphere, and the interior of roots. The γ -Proteobacteria increased along the gradient toward the interior of the plant while the *Holophaga/Acidobacterium* group decreased.

Chelius & Triplett (26) discovered that the interior of maize roots is inhabited by six bacterial and two archaeal divisions. In agreement with Marilley & Aragno (74), the Proteobacteria dominated the interior of the root. Several independent isolations of *Klebsiella pneumoniae* have been made from the interior of maize roots (24, 25, 31, 93), also in agreement with the observation that γ -Proteobacteria are enriched in the plant interior (74). The cultured collection from maize roots was also diverse with members from four bacterial divisions, which included a new bacterial genus and species in the Flexibacter group, *Dyadobacter fermentans* (24, 25).

Clearly the plant species strongly influences rhizosphere bacterial diversity. However, much more remains to be done to understand these relationships including whether the origin of some rhizosphere bacteria may be the plant seed.

Transgenic Plants and Microbial Diversity

The microbial diversity of the rhizospheres of field-grown T4-lysozyme-expressing potatoes, control transgenic potatoes that possess only the marker gene, and non-transgenic parental potatoes were not significantly different as measured with 16S rDNA DGGE profiles. In addition, a T4-lysozyme-tolerant *Pseudomonad* used as an inoculum strain did not become dominant in the community of any of the rhizospheres, including the rhizospheres of the T4-lysozyme-expressing plants. As many bacteria are sensitive to T4-lysozyme, this was a surprising result. The half-life of T4-lysozyme in nonsterile soil may be so brief that the concentration of this protein never builds to a high enough level to affect the bacterial community. Similarly, the rhizosphere communities of Barnase/Barstar transgenic potato differed little from those of the nontransgenic parent plant (71).

Herbicide-tolerant transgenic canola and wheat plants can harbor a different bacterial community either in the rhizosphere or in the root interior compared to

nontransgenic varieties (33, 118, 119), but these differences cannot be attributed solely to the transgene because the plant varieties tested were not otherwise isogenic.

Bacterial Rhizosphere Communities and Plant Disease

Soils that suppress plant disease have been known for many years. However, the cause of the plant disease suppression in many cases remains unknown. One area in which much progress has been made is the study of natural suppression of “take-all,” a wheat root disease caused by the fungus *Gaeumannomyces graminis* var. *tritici*. This natural disease suppression, a phenomenon known as “take-all decline” (TAD) is manifested as a spontaneous reduction of disease after an extended period of barley or wheat monoculture. Many studies have reported the association of antibiotic-producing fluorescent *Pseudomonas* species with disease suppression [summarized by Raaijmakers et al. (96b, 96c)]. Further studies demonstrated that production of the antibiotic compound 2,4-diacetylphloroglucinol by fluorescent *Pseudomonas* spp. was a critical component of this natural disease suppression (96a, 96c). Inoculation with the causal agent of take-all disease of wheat, *Gaeumannomyces graminis* var. *tritici*, results in a noticeable increase in the culturable *Pseudomonads* in the rhizosphere bacterial community (80). Several other groups of bacteria increased in these rhizospheres as well. Some of these were cultured but were not able to significantly influence the growth of the fungal pathogen in vitro, but they did vary in their ability to be antagonistic toward a *Pseudomonas* strain suppressive toward the disease.

Yang et al. (140) discovered that the bacterial diversity of *Phytophthora*-infected avocado roots is much greater than that of uninfected roots. Roots inoculated with the disease-suppressive bacterium *Pseudomonas fluorescens* 513 were disease-free and had a rhizosphere community similar to that of plants not inoculated with the pathogen. A likely explanation of this result is that diseased roots probably release more nutrients into the rhizosphere as a result of their own decay. These increased nutrients attract bacteria that might not normally be competitive in the rhizosphere.

Bacterial Antibiotic Production and Rhizosphere Microbial Diversity

In the first culture-independent analysis of the effects of bacterial antibiotic production on a natural community, Robleto et al. (107) showed that the production of the peptide antibiotic by *Rhizobium etli* CE3(pT2TFXK) resulted in a dramatic reduction in the diversity of trifolitoxin-sensitive α -Proteobacteria in the rhizosphere of *Phaseolus vulgaris*. This reduction did not occur in the rhizosphere of plants inoculated with the isogenic, nontrifolitoxin-producing strain, *R. etli* CE3(pT2TX3K). None of the treatments caused a detectable decline in total bacterial diversity of the rhizosphere, which was expected, as most bacteria are trifolitoxin-resistant.

Glandorf et al. (43) engineered *Pseudomonas putida* WCS358r to produce the antifungal compound phenazine-1-carboxylic acid (PCA). The engineered strains

decreased the fungal diversity of the wheat rhizosphere more consistently and for a longer period.

Though the study by McSpadden Gardener et al. (80) indicates that the interaction of the fungal pathogen with the plant alters the diversity and composition of the wheat rhizosphere, the 2,4-diacetylphloroglucinol produced by *Pseudomonas* spp. recovered from such assays may play a role in restructuring the rhizosphere microbial community in the study discussed above (80). This compound is known to have antibacterial properties and has been recovered from soils naturally suppressive for take-all decline (96a).

Rhizosphere Microbial Diversity and Plant Nutrient Status

Few papers have examined the effects of mineral nutrient status on rhizosphere microbial diversity. Yang & Crowley (139) grew barley plants in low- and high-iron soils. Using amplified 16S rDNA from various locations on the roots and separated by DGGE, the authors found qualitative differences between the treatments but did not define which organisms comprised the differences. In a study of low-nitrogen conditions on bacterial diversity in the rhizosphere of bean plants, Schallmach et al. (113) found that low N increased the proportion of α -Proteobacteria relative to the entire bacterial population near the root tip. This may be caused by the accumulation of root-nodulating rhizobia near the root tip. Throughout the root, low-N status increased the proportion of high G + C gram-positive bacteria. Both studies used methodology that has inherently low resolution. As a result, differences between treatments were difficult to observe.

Epiphytic Bacterial Diversity

Little has been done to describe epiphytes from a culture-independent perspective. As with other environments, leaf surfaces are inhabited by a wide variety of bacteria not known in culture collections. But to date this has only been studied on citrus (141) and the seagrass *Halophia stipulacea* (133, 134).

NONMETHANOGENIC ARCHAEA IN SOIL

Organisms within the domain Archaea have been classified among three divisions: Crenarchaeota, Euryarchaeota, and Korarchaeota (7). Methanogens are Euryarchaeota from soil and were among the earliest organisms recognized as members of the Archaea (137). Many methanogens have been cultured and characterized. A culture-independent study of Archaea in a Finnish forest soil reported the presence of *Halobacterium*-like Euryarchaeota (61). To date, none of the Korarchaeota has been cultured, and the only culture-independent evidence of their existence comes from extreme environments, particularly hot springs (52, 104). Prior to their first report in a temperate soil, the Crenarchaeota were thought to be present only in extreme environments. Ueda et al. (129) first reported culture-independent evidence for Crenarchaeota in a nonextreme soil environment. Two

16S rDNA clones were sequenced and found to be phylogenetically most similar to 16S rDNA sequences of Crenarchaeotal origin. Their template DNA was derived from a soybean field in Japan. Using an Archaeal-specific primer for the amplification of 16S rDNA clones, Bintrim et al. (12) found a wider diversity of Crenarchaeota in a Wisconsin soil. Culture-independent evidence of Crenarchaeota in lake sediments, tropical soils, and agricultural soils from Germany, Indiana, Michigan, and Norway suggests that the diversity of Crenarchaeota in terrestrial environments is broad (15, 20, 48, 72, 84, 110, 114).

Although not one of these soil Crenarchaeota has been cultured, their abundance can be determined using molecular tools with some interesting results. The first suggestion of the abundance of Crenarchaeota in soil relative to the Bacteria came from a culture-independent analysis of 16S rDNA clones from two adjacent Amazonian soils (15), where just 2 of 100 clones were of Archaeal origin. A more thorough analysis of Crenarchaeal abundance in soil by probing total community rRNA with a Crenarchaeota-specific probe showed that their abundance in a native soil ($0.37\% \pm 0.13\%$) was much lower than in a cultivated soil ($1.42\% \pm 0.59\%$). Jurgens & Saano (62) found that soil Crenarchaeota are sensitive to deforestation. Different Crenarchaeota were found in the control uncut-forest soil compared to soils from forests that were recently cleared with or without prescribed burning. Although the Crenarchaeota in the cut-forest soils were different from the native soil, their phylogenetic diversity was much higher than in the native soil. Sandaa et al. (110) found that the abundance of Crenarchaeota declined from $1.3\% + 0.3\%$ of all DAPI-stained cells to a level that was below detection following heavy metal contamination of the soil.

Some of the work summarized in the above paragraph strongly suggests that the plant community in the soil may influence the diversity and abundance of soil Crenarchaeota. Simon et al. (120) showed root colonization of soil Crenarchaeota. Chelius & Triplett (26) presented the first evidence for the interior colonization of plant roots by Crenarchaeota and Euryarchaeota.

Clearly more work is needed on the interactions of Archaea in soil and plants, especially in regard to their roles in these environments. Rapid progress will require culturing of these organisms so that their metabolism and physiology can be assessed rigorously through mutagenesis.

FUTURE NEEDS IN UNDERSTANDING MICROBIAL DIVERSITY

Current methodologies struggle to describe the vast microbial diversity in soil. For this reason we have recently turned to studying freshwater microbial diversity, as the microbial diversity of this habitat is considerably less complex than is soil (38, 39). Our hope is that once we have learned to comprehensively analyze microbial diversity in lakes, we can then begin to understand how to scale up our methods to understand soil communities.

In the meantime, all methods continue to improve. In particular, genomic approaches can be expected to improve our understanding of the role of uncultured microbes in the environment. The first genomic libraries of environmental DNA were created by DeLong and coworkers (10, 125, 132). This work resulted in the discovery of a photoactive proteorhodopsin in marine organisms that might play an important role in phototrophy in oceans (9). Similar analyses of uncultured organisms are in progress with soil as the source of DNA. Rondon et al. (108) reported the construction of a BAC library containing inserts of soil microbial DNA. Brady et al. (17) prepared a cosmid library of soil DNA and identified and sequenced a gene cluster for the biosynthesis of violacein, a potent broad-spectrum antibiotic. These works illustrate the ability to use these libraries in the identification of new secondary products, which would be difficult to discover by first culturing the producing organism.

Genomics of environmental DNA can lead to the discovery of important physiological processes in uncultured microorganisms. Efforts are already underway in a number of laboratories to obtain a significant amount of genome sequence from uncultured soil organisms, particularly from the *Acidobacterium* group, which are so common in soils. These data will provide clues regarding the role of these organisms in soil. With enough sequence information, the metabolic pathways of these organisms can be constructed, leading to effective strategies for the culturing of these organisms. The sequence data will also permit the construction of microarrays containing all known open reading frames that can be used to determine gene expression over time and space in the environment.

However, given the large number of organisms in soil, the target DNA to be sequenced must be chosen with care. For example, although the *Acidobacterium* group appears to be common in soil, no one has yet correlated its presence or absence to any microbial process in soil or any chemical or physical properties in soil. Some strong correlations between the presence of an organism with microbial processes or soil structure and function would be helpful in making wise choices prior to making large investments in such sequencing projects. As a result, linkage of specific organisms to ecosystem function over time and space is fundamental work that must go forward if we are to understand the role of these uncultured organisms.

The field also needs more definitive descriptions of those organisms that appear or disappear with a given treatment. Seeing patterns of diversity changing with treatments using DGGE, TGGE, ARISA, or T-RFLP is less satisfying if no attempt is made to identify the organisms affected.

We have also found that snapshots of diversity where the microbial diversity is assessed at one time in one location is not terribly informative. Where diversity over time and space has been measured, the dynamic nature of microbial communities is observed and the presence or absence of individual populations can be compared with changes in ecosystem processes.

The future in this area is bright, particularly as methods and data analysis improve. What we know today is still very much dwarfed by what we do not know.

Systems approaches, whether they be at the level of genomics, ecosystem function, or biocomplexity, will no doubt bring us new insights over the next decades that cannot now be imagined.

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LITERATURE CITED

1. Akman L, Aksoy S. 2001. A novel application of gene arrays: *Escherichia coli* array provides insight into the biology of the obligate endosymbiont of tsetse flies. *Proc. Natl. Acad. Sci. USA* 98:7546–51
2. Amann R. 2000. Who is out there? Microbial aspects of biodiversity. *Syst. Appl. Microbiol.* 23:1–8
3. Amann R, Fuchs BM, Behrens S. 2001. The identification of microorganisms by fluorescence in situ hybridisation. *Curr. Opin. Biotechnol.* 12:231–36
4. Amann RI. 1995. Fluorescently labeled, ribosomal RNA-targeted oligonucleotide probes in the study of microbial ecology. *Mol. Ecol.* 4:543–53
5. Amann RI, Ludwig W, Schleifer KH. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59:143–69
6. Bahr M, Hobbie JE, Sogin ML. 1996. Bacterial diversity in an arctic lake: a freshwater SAR11 cluster. *Aquat. Microb. Ecol.* 11:271–77
7. Barns SM, Delwiche CF, Palmer JD, Pace NR. 1996. Perspectives on archaeal diversity, thermophily and monophyly from environmental rRNA sequences. *Proc. Natl. Acad. Sci. USA* 93:9188–93
8. Beaulieu M, Bécaert V, Deschênes L, Villemur R. 2000. Evolution of bacterial diversity during enrichment of PCP-degrading activated soils. *Microb. Ecol.* 40:345–55
9. Béjà O, Aravind L, Koonin EV, Suzuki MT, Hadd A, et al. 2000. Bacterial rhodopsin: evidence for a new type of phototrophy in the sea. *Science* 289:1902–6
10. Beja O, Suzuki MT, Koonin EV, Aravind L, Hadd A, et al. 2000. Construction and analysis of bacterial artificial chromosome libraries from a marine microbial assemblage. *Environ. Microbiol.* 2:516–29
11. Bertilsson A, Polz M. 2001. Application of a diversity array to study specific substrate utilization in individual populations of aquatic heterotrophic bacteria. *9th Int. Symp. Microb. Ecol.* Amsterdam, The Netherlands
12. Bintrim SB, Donohue TJ, Handelsman J, Roberts GP, Goodman RM. 1997. Molecular phylogeny of archaea from soil. *Proc. Natl. Acad. Sci. USA* 94:277–82
13. Borneman J. 1999. Culture-independent identification of microorganisms that

- respond to specified stimuli. *Appl. Environ. Microbiol.* 65:3398–400
14. Borneman J, Skroch PW, Osullivan KM, Palus JA, Rumjanek NG, et al. 1996. Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl. Environ. Microbiol.* 62:1935–43
 15. Borneman J, Triplett EW. 1997. Molecular microbial diversity in soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Appl. Environ. Microbiol.* 63:2647–53
 16. Bowman JP, McCammon SA, Rea SM, McMeekin TA. 2000. The microbial composition of three limnologically disparate hypersaline Antarctic lakes. *FEMS Microbiol. Lett.* 183:81–88
 17. Brady SF, Chao CJ, Handelsman J, Clardy J. 2001. Cloning and heterologous expression of a natural product biosynthetic gene cluster from eDNA. *Org. Lett.* 3:1981–84
 18. Braker G, Zhou J, Wu L, Devol AH, Tiedje JM. 2000. Nitrite reductase genes (*nirK* and *nirS*) as functional markers to investigate diversity of denitrifying bacteria in pacific northwest marine sediment communities. *Appl. Environ. Microbiol.* 66:2096–104
 19. Bruns MA. 1999. Comparative diversity of ammonia oxidizer 16S rRNA gene sequences in native, tilled, and successional soils. *Appl. Environ. Microbiol.* 65:2994–3000
 20. Buckley DH, Graber JR, Schmidt TM. 1998. Phylogenetic analysis of nonthermophilic members of the kingdom Crenarchaeota and their diversity and abundance in soils. *Appl. Environ. Microbiol.* 64:4333–39
 21. Buckley DH, Schmidt TM. 2001. The structure of microbial communities in soil and the lasting impact of cultivation. *Microb. Ecol.* 42:11–21
 22. Busse MD, Ratcliff AW, Shestak CJ, Powers RF. 2001. Glyphosate toxicity and the effects of long-term vegetation control on soil microbial communities. *Soil Biol. Biochem.* 33:1777–89
 23. Chandler DP, Fredrickson JK, Brockman J. 1997. Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. *Mol. Ecol.* 6:475–82
 24. Chelius MK, Triplett EW. 2000. *Dyadobacter fermentans* gen. nov., sp. nov., a novel gram-negative bacterium isolated from surface-sterilized *Zea mays* stems. *Int. J. Syst. Evol. Microbiol.* 50:751–58
 25. Chelius MK, Triplett EW. 2000. Immunolocalization of dinitrogenase reductase produced by *Klebsiella pneumoniae* in association with *Zea mays* L. *Appl. Environ. Microbiol.* 66:783–87
 26. Chelius MK, Triplett EW. 2001. The diversity of archaea and bacteria in association with the roots of *Zea mays* L. *Microb. Ecol.* 41:252–63
 27. Cho J-C, Tiedje JM. 2000. Biogeography and degree of endemism of fluorescent *Pseudomonas* strains in soil. *Appl. Environ. Microbiol.* 66:5448–56
 28. Degens BP, Harris JA. 1997. Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. *Soil Biol. Biochem.* 29:1309–20
 29. DeLong EE, Pace NR. 2001. Environmental diversity of Bacteria and Archaea. *Syst. Biol.* 50:470–78
 30. Dojka MA, Harris JK, Pace NR. 2000. Expanding the known diversity and environmental distribution of an uncultured phylogenetic division of bacteria. *Appl. Environ. Microbiol.* 66:1617–21
 31. Dong YM, Glasner JD, Blattner FR, Triplett EW. 2001. Genomic interspecies microarray hybridization: rapid discovery of three thousand genes in the maize endophyte, *Klebsiella pneumoniae* 342, by microarray hybridization with *Escherichia coli* K-12 open reading frames. *Appl. Environ. Microbiol.* 67:1911–21
 32. Dunbar J, Takala S, Barns SM, Davis

- JA, Kuske CR. 1999. Levels of bacterial community diversity in four arid soils compared by cultivation and 16S rRNA gene cloning. *Appl. Environ. Microbiol.* 65:1662–69
33. Dunfield KE, Xavier LJC, Germida JJ. 1999. Identification of *Rhizobium leguminosarum* and *Rhizobium* sp. (Cicer) strains using a custom fatty acid methyl ester (FAME) profile library. *J. Appl. Microbiol.* 86:78–86
34. El Fantroussi S, Verschuere L, Verstraete W, Top EM. 1999. Effect of phenylurea herbicides on soil microbial communities estimated by analysis of 16S rRNA gene fingerprints and community-level physiological profiles. *Appl. Environ. Microbiol.* 65:982–88
35. Farrelly V, Rainey FA, Stackebrandt E. 1995. Effect of genome size and *rrn* gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl. Environ. Microb.* 61:2798–801
36. Felske A, Wolterink A, Van Lis R, De Vos WM, Akkermans ADL. 2000. Response of a soil bacterial community to grassland succession as monitored by 16S rRNA levels of the predominant ribotypes. *Appl. Environ. Microbiol.* 66:3998–4003
37. Fernández A, Huang SY, Seston S, Xing J, Hickey R, et al. 1999. How stable is stable? Function versus community composition. *Appl. Environ. Microbiol.* 65:3697–704
38. Fisher MM, Klug JL, Lauster G, Newton M, Triplett EW. 2000. Effects of resources and trophic interactions on freshwater bacterioplankton diversity. *Microb. Ecol.* 40:125–38
39. Fisher MM, Triplett EW. 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. *Appl. Environ. Microbiol.* 65:4630–36
40. Garland JL, Mills AL. 1991. Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* 57:2351–59
41. Gaston KJ. 1996. *Biodiversity: A Biology of Numbers and Difference*. Oxford, UK: Blackwell Sci.
42. Gerhardt P, ed. 1981. *Manual of Methods for General Bacteriology*. Washington, DC: ASM
43. Glandorf DCM, Verheggen P, Jansen T, Jorritsma JW, Smit E, et al. 2001. Effect of genetically modified *Pseudomonas putida* WCS358r on the fungal rhizosphere microflora of field-grown wheat. *Appl. Environ. Microbiol.* 67:3371–78
44. Hadrys H, Balick M, Schierwater B. 1992. Application of random amplified polymorphic DNA (RAPD) in molecular ecology. *Mol. Ecol.* 1:55–63
45. Harris D. 1994. Analyses of DNA extracted from microbial communities. In *Beyond the Biomass*, ed. K Ritz, J Dighton, KE Giller, pp. 111–18. Chichester, UK: Wiley
46. Head IM, Saunders JR, Pickup RW. 1998. Microbial evolution, diversity, and ecology: a decade of ribosomal RNA analysis of uncultivated microorganisms. *Microb. Ecol.* 35:1–21
47. Henckel T, Friedrich M, Conrad R. 1999. Molecular analyses of the methane-oxidizing microbial community in rice field soil by targeting the genes of the 16S rRNA, particulate methane monooxygenase, and methanol dehydrogenase. *Appl. Environ. Microbiol.* 65:1980–90
48. Hershberger KL, Barns SM, Reysenbach AL, Dawson SC, Pace NR. 1996. Wide diversity of Crenarchaeota. *Nature* 384:420
49. Heuer H, Smalla K. 1997. Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In *Modern Soil Microbiology*, ed. JD van Elsas, EMH Wellington,

- JT Trevors, pp. 353–74. New York: Marcel Dekker
50. Heuer H, Smalla K. 1997. Evaluation of community-level catabolic profiling using BIOLOG GN microplates to study microbial community changes in potato phyllosphere. *J. Microbiol. Methods* 30:49–61
 51. Hiorns WD, Methe BA, Nierzwicki-Bauer SA, Zehr JP. 1997. Bacterial diversity in Adirondack Mountain lakes as revealed by 16S rRNA gene sequences. *Appl. Environ. Microbiol.* 63:2957–60
 52. Hjorleifsdottir S, Skirnisdottir S, Hreggvidsson GO, Holst O, Kristjansson JK. 2001. Species composition of cultivated and noncultivated bacteria from short filaments in an Icelandic hot spring at 88 degrees C. *Microb. Ecol.* 42:117–25
 53. Höfle MG, Haas H, Dominik K. 1999. Seasonal dynamics of bacterioplankton community structure in a eutrophic lake as determined by 5S rRNA analysis. *Appl. Environ. Microbiol.* 65:3164–74
 54. Holben WE, Harris D. 1995. DNA-based monitoring of total bacterial community structure in environmental samples. *Mol. Ecol.* 4:627–31
 55. Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. 1994. *Bergey's Manual of Determinative Bacteriology*. Baltimore, MD: Wilkins & Wilkins
 56. Hughes JB, Hellmann JJ, Ricketts TH, Bohannon BJM. 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. *Appl. Environ. Microbiol.* 67:4399–406
 57. Ibekwe AM, Kennedy AC. 1998. Fatty acid methyl ester (FAME) profiles as a tool to investigate community structure of two agricultural soils. *Plant Soil* 206:151–61
 58. Ibekwe AM, Papiernik SK, Gan J, Yates SR, Yang CH, Crowley DE. 2001. Impact of fumigants on soil microbial communities. *Appl. Environ. Microbiol.* 67:3245–57
 59. Insam H. 1997. Substrate utilization tests in microbial ecology—a preface to the special issue of the *Journal of Microbiological Methods*. *J. Microbiol. Methods* 30:1–2
 - 59a. Jensen S, Øvreås L, Daae FL, Torsvik V. 1998. Diversity in methane enrichments from agricultural soil revealed by DGGE separation of PCR amplified 16S rDNA fragments. *FEMS Microbiol. Ecol.* 26:17–26
 60. Johnsen K, Jacobsen CS, Torsvik V, Sørensen J. 2001. Pesticide effects on bacterial diversity in agricultural soils—a review. *Biol. Fertil. Soils* 33:443–53
 61. Jurgens G, Lindstrom K, Saano A. 1997. Novel group within the kingdom Crenarchaeota from boreal forest soil. *Appl. Environ. Microbiol.* 63:803–5
 62. Jurgens G, Saano A. 1999. Diversity of soil Archaea in boreal forest before, and after clear-cutting and prescribed burning. *FEMS Microbiol. Ecol.* 29:205–13
 63. Kaiser O, Pühler A, Selbitschka W. 2001. Phylogenetic analysis of microbial diversity in the rhizoplane of oilseed rape (*Brassica napus* cv. Westar) employing cultivation-dependent and cultivation-independent approaches. *Microb. Ecol.* 42:136–49
 64. Karner M, Fuhrman JA. 1997. Determination of active marine bacterioplankton: a comparison of universal 16S rRNA probes, autoradiography, and nucleoid staining. *Appl. Environ. Microbiol.* 63:1208–13
 65. Kitts CL. 2001. Terminal restriction fragment patterns: a tool for comparing microbial communities and assessing community dynamics. *Curr. Issues Intest. Microbiol.* 2:17–25
 66. Lane DJ. 1991. 16S/23S rRNA sequencing. In *Nucleic Acid Techniques in Bacterial Systematics*, ed. E Stackebrandt, M Goodfellow, pp. 131–73. Chichester, UK: Wiley
 67. Lawlor K, Knight BP, Barbosa-Jefferson VL, Lane PW, Lilley AK, et al. 2000. Comparison of methods to investigate

- microbial populations in soils under different agricultural management. *FEMS Microbiol. Ecol.* 33:129–37
68. Liesack W, Stackebrandt E. 1992. Occurrence of novel groups of the domain bacteria as revealed by analysis of genetic material isolated from an Australian terrestrial environment. *J. Bacteriol.* 174:5072–78
69. Liu J, Dazzo FB, Glagoleva O, Yu B, Jain AK. 2001. CMEIAS: a computer-aided system for the image analysis of bacterial morphotypes in microbial communities. *Microb. Ecol.* 41:173–94
70. Liu WT, Marsh TL, Cheng H, Forney LJ. 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* 63:4516–22
71. Lukow T, Dunfield PF, Liesack W. 2000. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. *FEMS Microbiol. Ecol.* 32:241–47
72. MacGregor BJ, Moser DP, Alm EW, Nealson KH, Stahl DA. 1997. Crenarchaeota in Lake Michigan sediment. *Appl. Environ. Microbiol.* 63:1178–81
73. Magurran AE. 1988. *Ecological Diversity and Its Measurement*. Princeton, NJ: Princeton Univ. Press
74. Marilley L, Aragno M. 1999. Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. *Appl. Soil Ecol.* 13:127–36
75. Marschner P, Yang C-H, Lieberei R, Crowley DE. 2001. Soil and plant specific effects on bacterial community composition in the rhizosphere. *Soil Biol. Biochem.* 33:1437–45
76. Marsh T. 1999. Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* 2:323–27
77. Marsh TL, Saxman P, Cole J, Tiedje J. 2000. Terminal restriction fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Appl. Environ. Microbiol.* 66:3616–20
78. Massol-Deya AA, Odelson DA, Hickey RF, Tiedje JM. 1995. Bacterial community fingerprinting of amplified 16S and 16-23S ribosomal DNA gene sequences and restriction endonuclease analysis (ARDRA). In *Molecular Microbial Ecology Manual*, ed. ADL Akkermans, JD van Elsas, FJ de Bruijn, 2:1–8. Dordrecht/Boston/London: Kluwer
79. McCaig AE, Glover LA, Prosser JI. 1999. Molecular analysis of bacterial community structure and diversity in unimproved and improved upland grass pastures. *Appl. Environ. Microbiol.* 65:1721–30
80. McSpadden Gardener B, Schroeder K, Kalloger S, Raaijmakers J, et al. 2000. Genotypic and phenotypic diversity of pHLI-containing *Pseudomonas* isolated from the rhizosphere of wheat. *Appl. Environ. Microbiol.* 66:1939–46
81. Mergel A, Schmitz O, Mallman T, Bothe H. 2001. Relative abundance of denitrifying and dinitrogen-fixing bacteria in layers of a forest soil. *FEMS Microbiol. Ecol.* 36:33–42
82. Murray AE, Lies D, Li G, Nealson K, Zhou J, Tiedje JM. 2001. DNA/DNA hybridization to microarrays reveals gene-specific differences between closely related microbial genomes. *Proc. Natl. Acad. Sci. USA* 98:9853–58
83. Muyzer GA, de Waal EC, Uitterlinden AG. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59:695–700
84. Nakatsu CH, Torsvik V, Øvreås L. 2000.

- Soil community analysis using DGGE of 16S rDNA polymerase chain reaction products. *Soil Sci. Soc. Am. J.* 64:1382–88
85. Nicholson PS, Hirsch PR. 1998. The effects of pesticides on the diversity of culturable soil bacteria. *J. Appl. Microbiol.* 84:551–58
 86. Niemi RM, Heiskanen I, Wallenius K, Lindstrom K. 2001. Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. *J. Microbiol. Methods* 45:155–65
 87. Normander B, Prosser JI. 2000. Bacterial origin and community composition in the barley phytosphere as a function of habitat and presowing conditions. *Appl. Environ. Microbiol.* 66:4372–77
 88. Nüsslein K, Tiedje JM. 1999. Soil bacterial community shift correlated with change from forest to pasture vegetation in a tropical soil. *Appl. Environ. Microbiol.* 65:3622–26
 89. Osborne AM, Moore ERB, Timmis KN. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ. Microbiol.* 2:39–50
 90. Øvreås L. 2000. Population and community level approaches for analysing microbial diversity in natural environments. *Ecol. Lett.* 3:236–51
 91. Øvreås L, Torsvik V. 1998. Microbial diversity and community structure in two different agricultural soil communities. *Microb. Ecol.* 36:303–15
 92. Pace NR. 2000. Community interactions: towards a natural history of the microbial world. *Environ. Microbiol.* 2: 7–8
 93. Palus JA, Borneman J, Ludden PW, Triplett EW. 1996. A diazotrophic bacterial endophyte isolated from stems of *Zea mays* L and *Zea luxurians* Iltis and Doebley. *Plant Soil* 186:135–42
 94. Pickup R. 1991. Detection and study of microorganisms in the environment—new approaches. 8:499–503
 95. Poly F, Ranjard L, Nazaret S, Gourbiere F, Monrozier LJ. 2001. Comparison of *nifH* gene pools in soils and soil microenvironments with contrasting properties. *Appl. Environ. Microbiol.* 67:2255–62
 96. Posch T, Pernthaler J, Alfreider A, Psenner R. 1997. Cell-specific respiratory activity of aquatic bacteria studied with the tetrazolium reduction method, cyto-clear slides, and image analysis. *Appl. Environ. Microbiol.* 63:867–73
 - 96a. Raaijmakers JM, Bonsall RE, Weller DM. 1999. Effect of population density of *Pseudomonas fluorescens* on production of 2,4-diacetylphloroglucinol in the rhizosphere of wheat. *Phytopathology* 89:470–75
 - 96b. Raaijmakers JM, Weller DM. 1998. Natural plant protection by 2,4-diacetylphloroglucinol-producing *Pseudomonas* spp. in take-all decline soils. *Mol. Plant-Microb. Interact.* 11:144–52
 - 96c. Raaijmakers JM, Weller DM, Thoma-show LS. 1997. Frequency of antibiotic-producing *Pseudomonas* spp. in natural environments. *Appl. Environ. Microbiol.* 63:881–87
 97. Rainey FA, Ward N, Sly LI, Stackebrandt E. 1994. Dependence on the taxon composition of clone libraries for PCR amplified, naturally occurring 16S rDNA, on the primer pair and the cloning system used. *Experientia* 50:796–97
 98. Rainey FA, Ward-Rainey NL, Stackebrandt E. 1996. *Clostridium paradoxum* DSM 7308T contains multiple 16S rRNA genes with heterogeneous intervening sequences. *Microbiology* 142: 2087–91
 99. Ramirez-Saad HC, Sessitsch A, de Vos WM, Akkermans ADL. 2000. Bacterial community changes and enrichment of *Burkholderia*-like bacteria induced by chlorinated benzoates in a peat-forest soil-microcosm. *Syst. Appl. Microbiol.* 23:591–98

100. Ranjard L, Brothier E, Nazaret S. 2000. Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. *Appl. Environ. Microbiol.* 66:5334–39
101. Ranjard L, Poly F, Combrisson J, Richaume A, Gourbiere F, et al. 2000. Heterogeneous cell density and genetic structure of bacterial pools associated with various soil microenvironments as determined by enumeration and DNA fingerprinting approach (RISA). *Microb. Ecol.* 39:263–72
102. Ranjard L, Poly F, Lata JC, Mougél C, Thioulouse J, Nazaret S. 2001. Characterization of bacterial and fungal soil communities by automated ribosomal intergenic spacer analysis fingerprints: biological and methodological variability. *Appl. Environ. Microbiol.* 67:4479–87
103. Ranjard L, Richaume AS. 2001. Quantitative and qualitative microscale distribution of bacteria in soil. *Res. Microbiol.* 152:707–16
104. Reysenbach AL, Ehringer H, Hershberger K. 2000. Microbial diversity at 83 degrees C in Calcite Springs, Yellowstone National Park: another environment where the aquificales and “Korarchaeota” coexist. *Extremophiles* 4: 61–67
105. Reysenbach AL, Giver LJ, Wickham GS, Pace NR. 1992. Differential amplification of rRNA genes by polymerase chain reaction. *Appl. Environ. Microbiol.* 58:3417–18
106. Ritchie NJ, Schutter ME, Dick RP, Myrold DD. 2000. Use of length heterogeneity PCR and fatty acid methyl ester profiles to characterize microbial communities in soil. *Appl. Environ. Microbiol.* 66:1668–75
107. Robleto EA, Borneman J, Triplett EW. 1998. Effects of bacterial antibiotic production on rhizosphere microbial communities from a culture-independent perspective. *Appl. Environ. Microbiol.* 64: 5020–22
108. Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, et al. 2000. Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. *Appl. Environ. Microbiol.* 66:2541–47
109. Sandaa R-A, Torsvik V, Enger Ø. 2001. Influence of long-term heavy-metal contamination on microbial communities in soil. *Soil Biol. Biochem.* 33:287–95
110. Sandaa R-A, Torsvik V, Enger Ø, Daae FL, Castberg T, et al. 1999. Analysis of bacterial communities in heavy metal-contaminated soils at different levels of resolution. *FEMS Microbiol. Ecol.* 30: 237–51
111. Scala DJ, Kerkhof LJ. 1999. Diversity of nitrous oxide reductase (*nosZ*) genes in continental shelf sediments. *Appl. Environ. Microb.* 65:1681–87
112. Scala DJ, Kerkhof LJ. 2000. Horizontal heterogeneity of denitrifying bacterial communities in marine sediments by terminal restriction fragment length polymorphism analysis. *Appl. Environ. Microbiol.* 66:1980–86
113. Schallmach E, Minz D, Jurkevitch E. 2000. Culture-independent detection of changes in root-associated bacterial populations of common bean (*Phaseolus vulgaris* L.) following nitrogen depletion. *Microb. Ecol.* 40:309–16
114. Schleper C, Holben W, Klenk HP. 1997. Recovery of Crenarchaeotal ribosomal DNA sequences from freshwater-lake sediments. *Appl. Environ. Microbiol.* 63: 321–23
115. Schmalenberger A, Schwieger F, Tebbe CC. 2001. Effect of primers hybridizing to different evolutionarily conserved regions of the small-subunit rRNA gene in PCR-based microbial community analyses and genetic profiling. *Appl. Environ. Microbiol.* 67:3557–63
116. Schwieger F, Tebbe CC. 1998. A new approach to utilize PCR-single-strand

- conformation polymorphism for 16s rRNA gene-based microbial community analysis. *Appl. Environ. Microbiol.* 64: 4870–76
117. Sessitsch A, Weilharter A, Gerzabek MH, Kirchmann H, Kandeler E. 2001. Microbial population structures in soil particle size fractions of a long-term fertilizer field experiment. *Appl. Environ. Microbiol.* 67:4215–24
118. Siciliano SD, Germida JJ. 1999. Taxonomic diversity of bacteria associated with the roots of field-grown transgenic *Brassica napus* cv. Quest, compared to the non-transgenic *B. napus* cv. Excel and *B. rapa* cv. Parkland. *FEMS Microbiol. Ecol.* 29:263–72
119. Siciliano SD, Theoret CM, de Freitas JR, Hucl PJ, Germida JJ. 1998. Differences in the microbial communities associated with the roots of different cultivars of canola and wheat. *Can. J. Microbiol.* 44: 844–51
120. Simon HM, Dodsworth JA, Goodman RM. 2000. Crenarchaeota colonize terrestrial plant roots. *Environ. Microbiol.* 2:495–505
121. Small J, Call DR, Brockman FJ, Straub TM, Chandler DP. 2001. Direct detection of 16S rRNA in soil extracts by using oligonucleotide microarrays. *Appl. Environ. Microbiol.* 67:4708–16
122. Smalla K, Wachtendorf U, Liu W-T, Forney L. 1998. Analysis of BIOLOG GN substrate utilization patterns by microbial communities. *Appl. Environ. Microbiol.* 64:1220–25
- 122a. Smalla K, Wieland G, Buchner A, Zock A, Parzy J, et al. 2001. Bulk and rhizosphere soil bacterial communities studied by denaturing gradient gel electrophoresis: plant-dependent enrichment and seasonal shifts revealed. *Appl. Environ. Microbiol.* 67:4742–51
123. Smit E, Leeftang P, Gommans S, van den Broek J, van Mil S, Wernars K. 2001. Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. *Appl. Environ. Microbiol.* 67: 2284–91
124. Stackebrandt E, Liesack W, Goebel BM. 1993. Bacterial diversity in a soil sample from a subtropical Australian environment as determined by 16S rDNA analysis. *FASEB J.* 7:232–36
125. Stein JL, Marsh TL, Wu KY, Shizuya H, DeLong EF. 1996. Characterization of uncultivated prokaryotes: isolation and analysis of a 40-kilobase-pair genome fragment from a planktonic marine archaeon. *J. Bacteriol.* 178:591–99
126. Suzuki MT, Giovannoni SJ. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* 62:625–30
127. Tiedje JM, Asuming-Brempong S, Nusslein K, Marsh TL, Flynn SJ. 1999. Opening the black box of soil microbial diversity. *Appl. Soil Ecol.* 13:109–22
128. Torsvik V, Salte K, Sorheim R, Goksoyr J. 1990. Comparison of phenotypic diversity and DNA heterogeneity in a population of soil bacteria. *Appl. Environ. Microbiol.* 56:776–81
129. Ueda T, Suga Y, Matsuguchi T. 1995. Molecular phylogenetic analysis of a soil microbial community in a soybean field. *Eur. J. Soil Sci.* 46:415–21
130. van Elsas JD, Duarte GF, Rosado AS, Smalla K. 1998. Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. *J. Microb. Methods* 32:133–54
131. Vergin KL, Rappe MS, Giovannoni SJ. 2001. Streamlined method to analyze 16S rRNA gene clone libraries. *Biotechniques* 30:938–42
132. Vergin KL, Urbach E, Stein JL, DeLong EF, Lanol BD, Giovannoni SJ. 1998. Screening of a fosmid library of marine environmental genomic DNA fragments

- reveals four clones related to members of the order Planctomycetales. *Appl. Environ. Microbiol.* 64:3075–78
133. Weidner S, Arnold W, Puhler A. 1996. Diversity of uncultured microorganisms associated with the seagrass *Halophila stipulacea* estimated by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. *Appl. Environ. Microbiol.* 62:766–71
134. Weidner S, Arnold W, Stackebrandt E, Puhler A. 2000. Phylogenetic analysis of bacterial communities associated with leaves of the seagrass *Halophila stipulacea* by a culture-independent small-subunit rRNA gene approach. *Microb. Ecol.* 39:22–31
135. Wilson MJ, Weightman AJ, Wade WG. 1997. Applications of molecular ecology in the characterization of uncultured microorganisms associated with human disease. *Rev. Med. Microbiol.* 8:91–101
136. Woese C. 1987. Bacterial evolution. *Microbiol. Rev.* 51:221–71
137. Woese CR, Kandler O, Wheelis ML. 1990. Towards a natural system of organisms—proposal for the domains Archaea, Bacteria, and Eucarya. *Proc. Natl. Acad. Sci. USA* 87:4576–79
138. Xia XQ, Bollinger J, Ogram A. 1995. Molecular genetic analysis of the response of three soil microbial communities to the application of 2,4-D. *Mol. Ecol.* 4:17–28
139. Yang C-H, Crowley DE. 2000. Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. *Appl. Environ. Microbiol.* 66:345–51
140. Yang C-H, Crowley DE, Menge JA. 2001. 16S rDNA fingerprinting of rhizosphere bacterial communities associated with healthy and *Phytophthora* infected avocado roots. *FEMS Microbiol. Ecol.* 35:129–36
141. Yang C-H, Crowley DE, Borneman J, Keen NT. 2001. Microbial phyllosphere populations are more complex than previously realized. *Proc. Natl. Acad. Sci. USA* 98:3889–94
142. Yang Y-H, Yao J, Hu S, Qi Y. 2000. Effects of agricultural chemicals on DNA sequence diversity of soil microbial community: a study with RAPD marker. *Microb. Ecol.* 39:72–79
143. Yin B, Crowley D, Sparovek G, De Melo WJ, Borneman J. 2000. Bacterial functional redundancy along a soil reclamation gradient. *Appl. Environ. Microbiol.* 66:4361–65
144. Zhou J, Davey ME, Figueras JB, Rivkina E, Gilichinsky D, Tiedje JM. 1997. Phylogenetic diversity of a bacterial community determined from Siberian tundra soil DNA. *Microbiology* 143:3913–19