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Opening the black box of soil microbial diversity

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Abstract

Soil probably harbours most of our planet's undiscovered biodiversity. Recent results from both, culturing and nucleic acid-based approaches indicate that soil microbial diversity is even higher than previously imagined. One reason for the high diversity is that much of the diversity can be found at very small scales. If the same genotypes are not repeated at other locations, the large-scale diversity is greatly multiplied. It remains to be seen to what extent this large genotypic diversity actually affects functional diversity, microbial ecology, or biotechnological significance. Here we present a framework of methods for opening the soil black box that provides different levels of resolution of both microbial community structure and activity. The rationale for and examples of use of three of these methods are presented: guanine plus cytosine content of total soil DNA (G+C), terminal restriction fragment length polymorphism (T-RFLP) of 16S rRNA genes amplified from soil DNA, and amplified ribosomal DNA restriction analysis (ARDRA) of rRNA genes from soil DNA and from isolates. These methods give coarse and moderate scale resolution of the soil community. The G+C method, which is one of the few comprehensive coarse scale methods, is also quantitative and can be used to separate DNA into G+C fractions for a second level of composition or activity analysis. The example of the ARDRA method used here illustrates that the same populations of 2,4-D degraders became dominant in three soils of very different land use history and that several of the 2,4-D degrading isolates from these sites had the same ARDRA pattern found from the soil DNA indicating that the isolates represent the dominant populations in the 2,4-D treated soil.

Keywords: Soil DNA; SSU rRNA; Molecular ecology; Terminal restriction fragment length polymorphism (T-RFLP); Amplified ribosomal DNA restriction analysis (ARDRA)

1. Introduction

Research in soil microbiology over the last three decades has largely focused on analysis of microbial processes, with a notable exception being those efforts focused on Rhizobium ecology. The understanding of the populations responsible for these processes has been relatively neglected, yet it is these populations that are the fundamental units responsible for the processes. The next level of understanding of microbial processes should be at the organism level, e.g. from population dynamics through functional diversity to the molecular regulation of the key processes. Research at this level has, however, largely not been tractable, but with the rapid development of the tools of molecular biology, new efforts are now feasible.

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Understanding the dynamics of microbial communities is at the heart of contemporary microbial ecology, and understanding of the soil microbial community is probably the most challenging because of the exceptionally high microbial diversity in soil (Torsvik et al., 1990; Borneman et al., 1996; Kruske et al., 1997; Zhou et al., 1998; Nüsslein and Tiedje, 1998) and the complex and variable matrix in which soil microbes are embedded. Nonetheless, it is time to open the soil black box.

The central microbial community question is: What controls the distribution and abundance of members of the soil microbial community? And, how do these communities change with time in response to their environment? Major factors thought to control community composition are:

(i) the major resources for growth, e.g. different types of carbon from plant litter, rhizosphere or invertebrates;
(ii) different chemistries of the soil environment, especially pH and key nutrients, N, P, Fe;
(iii) factors which affect organism dispersal, such as soil structure, microaggregate stability, routes of dispersal; and
(iv) causes of population turnover, e.g. nematode and protozoan grazing, controls on lytic enzymes, protective soil matrices.

The major microbial processes of importance to terrestrial ecosystem function and global sustainability are a result of microbial cell growth, death, or the enzymatic function of non-growing cells. Methods that reveal the composition of microbial communities can then be applied over time and space in response to different environmental conditions to understand the linkages between key populations and processes. Once the tools for community analysis are available they can be applied to almost any question addressing the soil microbial state and function (Tiedje, 1995).

In this paper, we report on some of the major molecular methods that can be used to open the soil-microbial black box and illustrate their use. These methods can be grouped into those that measure the members present (structure) and those that provide some measure of activity (Fig. 1). Methods can also be positioned according to level of taxonomic hierarchy at which they resolve differences (Fig. 1, vertical scale). Some questions are adequately addressed at a coarse level of resolution, while others require a fine scale of resolution. The coarse scale usually samples the entire community, i.e. it is comprehensive, while methods for fine scale resolution often require analysis of target populations only in order to achieve the fine-scale resolution. A more complete listing of bacterial taxonomic methods

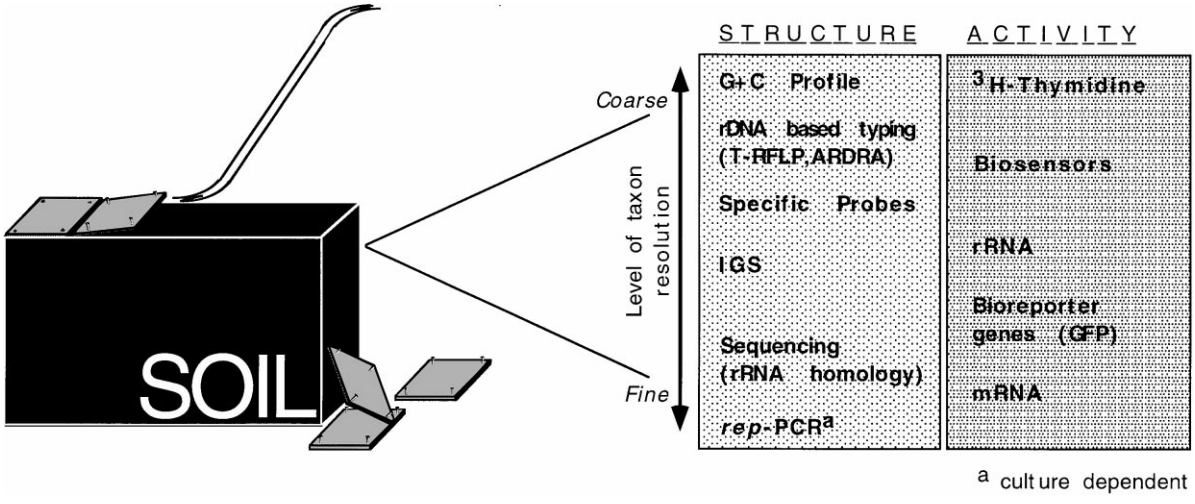

Fig. 1. New methods of soil microbial analysis break open the 'black box' and reveal new insight into the structure and activity of soil microbial communities.

and their level of taxonomic resolution can be found in Vandamme et al. (1996). Use of a few of the methods listed in Fig. 1 in soil ecology studies are illustrated in the following.

2. G+C analysis

2.1. Rationale for the method

Analysis of the guanine plus cytosine (G + C) content of DNA is useful when a coarse level of resolution is meaningful. This technique is based on the fact that prokaryotic DNA varies in G + C content from ca. 24% to 76% G + C vs. A + T, and that particular taxonomic groups only include organisms that vary in G + C content by no more than 3–5% (Goodfellow and O'Donnell, 1993; Vandamme et al., 1996). Hence, G + C can be related to taxonomy. The G + C content of some of the common soil bacteria are summarized by Holben and Harris (1995). The G + C method is a coarse measure of resolution since several taxonomic groups can share the same G + C range. There is, however, a general correlation with physiology; organisms of high G + C content, 60–75%, are generally obligate aerobes with oxidative metabolism, while organisms with fermentative metabolism largely have low G + C contents (Santo Domingo et al., 1998).

In microbiology, most methodologies resolve at the medium-to-fine-scale resolution, i.e. genus to species to subspecies level (Vandamme et al., 1996). Hence, the G + C method fills a gap in the tool box by providing one of the few coarse-level methods, especially for community analysis. Other advantages of the G + C method are: it is comprehensive for all DNA extracted; it is not subject to the biases of PCR-based methods; and portions of the DNA are not missed in analysis due to ineffective hybridization or similar losses in the analysis. The method is also quantitative, which is not true for many molecular methods now popular in microbial ecology, and it provides a means for uncovering rarer members of the community since it separates low biomass fractions from those reflecting dominant biomass. The disadvantages are that the method requires a reasonably large amount of DNA, e.g. 50 μg, and requires an ultracentrifuge to separate the G + C fractions.

2.2. Basic methodology

The method is described in detail by Holben and Harris (1995), the primary reference. The separation by base composition is based on the principle that bis-benzimidazole (Hoechst reagent number 33258) binds to adenine and thymidine and changes the buoyant density of DNA in proportion to its A + T (hence, G + C) content. A gradient of DNA fragments of different G + C concentrations is then established by equilibrium density-gradient (CsCl) centrification. After the gradient is established, fractions as small as 0.2 ml can be collected with a fraction collector. The DNA in each fraction is quantified by spectrophotometry and its G + C content is established by using a standard curve relating G + C content to density measured by a refractometer. Since the DNA fragments coming from soil DNA extraction methods are often 40–50 kb, the separation procedure reflects the G + C content of these sized fragments, which typically is the content of the entire organism and not influenced by shorter sequences which can have different G + C content reflecting recent gene transfer or conserved genes, e.g. rDNA. Recent work has shown that rRNA sequences found in particular G + C fractions provide a phylogeny consistent with the G + C content expected for that genus or species (Nüsslein and Tiedje, 1998). This confirms that the method separates taxa as predicted by G + C percentage.

The entire G + C separation process takes about four days, of which one is required for the equilibrium centrifugation. Several samples can be processed at the same time according to the capacity of the ultracentrifuge rotor. Profiles of the amount of DNA of particular G + C content can be compared among different samples to determine how different the communities are at this level of resolution (Fig. 2(a)). If they are not different, which is often the case for soils that have not experienced major environmental changes, community differences often can be detected by a secondary analysis of the fractionated DNA. The dye bis-benzimidazole is removed prior to secondary analyses by five repeated extractions of the DNA fractions in CsCl-saturated isopropanol, followed by spin-column chromatography (Wizard PCR preps, Promega, Madison, WI) with two washing steps (Nüsslein and Tiedje, 1998).

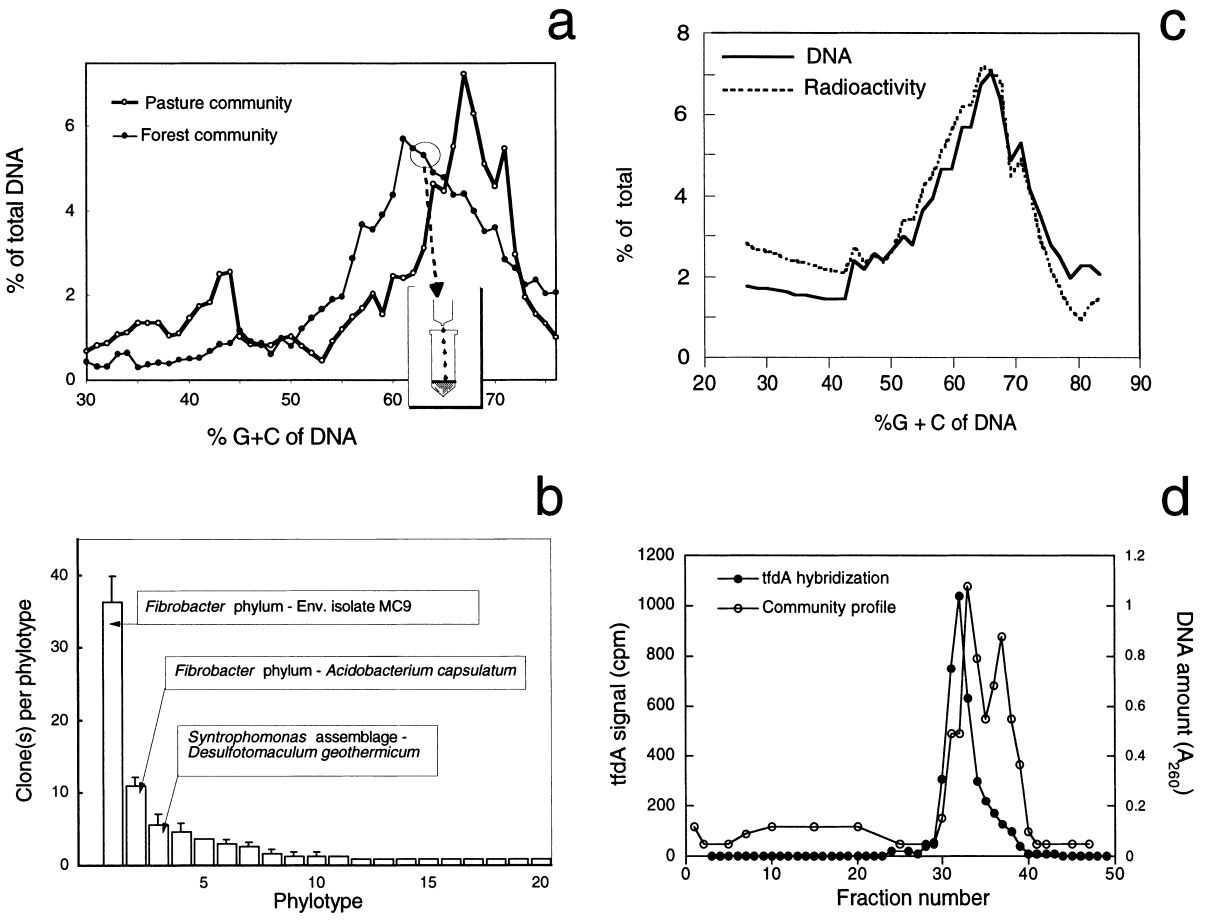

Fig. 2. Fractionation of soil DNA by its G + C content and various applications of the method. Panel 'a' shows the amount of DNA according to G + C content from adjacent soils, one under native forest and the other under pasture which had replaced the forest. The 63% G + C fraction was collected and the DNA used as target for PCR amplification of eubacterial 16S rRNA genes. Panel 'b' shows the abundance of unique phylotypes determined by restriction digestion of these PCR products. The identity of the ribosomal sequence most similar to the sequence of the three most abundant clones is shown. Panel 'c' shows the amount of ^{3}H-thymidine taken up in DNA of particular G + C content after a short-term incubation with the labeled substrate prior to DNA extraction. Panel 'd' shows which G + C fractions hybridized to the probe for the first gene (*tfd*A) in the 2,4-D degradation pathway. Data in panel 'b' redrawn from Nüsslein and Tiedje, 1999, in panel 'c' redrawn from Harris, 1994, and in panel 'd' redrawn from Holben et al., 1993.

2.3. Results

Several uses of the G + C method are illustrated in Fig. 2. In Fig. 2(a), the influence of forest vs. pasture vegetation on the composition of the soil community is shown. At this location, a portion of the native forest had been cleared for pasture ca. 80 years ago, but the soil parent material, slope and climate were identical (Nüsslein and Tiedje, 1999). The only management difference was that cattle roamed the pasture and wild pigs the forest. The soils were young volcanic ash soils from the Big Island of Hawaii. Replicate G + C profiles from paired samples taken at the same sites essentially showed the same profile, demonstrating the reproducibility of the method (Nüsslein and Tiedje, 1999). If the DNA content reflects biomass, which is likely, the shift in vegetation to pasture resulted in at least a 49% change in the microbial composition, since this is the area of the pasture profile that lies outside of the forest profile. Of course, organisms

contributing DNA of the same G + C content to the peak area could be unrelated, while those of different G + C content must be different species.

One type of secondary analysis coupled to the G + C method is to analyze the 16S rRNA genes found in particular separated fractions. The G + C separation resolves some of the diversity, hence each fraction should be made up of simpler rRNA and other gene populations. In Fig. 2(b), we found the abundance profile of 16S rRNA genes amplified by eubacterial primers from the 63% G + C fraction of the Hawaiian forest soil. Each unit on the *x*-axis represents a different operational taxonomic unit (OTU) which is distinguished by differences in 16S rDNA-PCR products revealed by cutting each clone with two pairs of tetrameric restriction endonucleases (Moyer et al., 1994). The first three band patterns were seen in several clones and, hence, may represent more abundant populations (OTUs) in the 63% G + C fraction, which also is a more dominant component of the total DNA. Clones of selected OTUs can then be sequenced for an indication of taxonomic identity. In this case, clones of three dominant OTUs were fully sequenced and two were found to be closely related and to be members of the newly recognized *Acidobacterium* assemblage (Nüsslein and Tiedje, 1999). *Acidobacterium* has only a few cultured members, but sequences from this group are becoming widely found in soil (Ludwig et al., 1997). Presumably, organisms of this assemblage are some of the dominant members of soil communities. This organism, however, was not found in the 63% fraction of the pasture soil (Nüsslein and Tiedje, 1999), confirming that some organisms of the same G + C content, but from different vegetative sites, were in fact also different.

The G + C method can also be used in conjunction with radioactive tracers to determine which fractions of the microbial community are active in DNA synthesis. Fig. 2(c) shows an experiment by Harris (1994) in which agricultural soil was first incubated with ^{3}H-thymidine for 2 h before DNA extraction and separation. The amount of radioactivity in each fraction was then measured. The result shows that all fractions were active in proportion to their biomass. This may not be the case in all soils, especially those that have experienced a severe stress or those receiving a new carbon source which may have stimulated a portion of the community.

DNA from the separated fractions can also be attached to blots and the panel of blotted DNA hybridized to DNA probes of various types, e.g. functional gene probes. Fig. 2(d) shows the work of Holben et al. (1993) in which DNA from a Saskatchewan agricultural soil, that was treated with 2,4-D for 40 years then enriched with more 2,4-D (Holben et al., 1992), was separated by G + C fractionation. The separated DNA placed on blots was then hybridized with ^{32}P-labeled tfdA gene. The tfdA gene codes for a dioxygenase whose only known function is to cleave the aryl–ether bond of 2,4-D, the first step in the 2,4-D degradation pathway (Fukumori and Hausinger, 1993). The peak of hybridization was on the left shoulder of the major biomass fraction and indicates the G + C content of the community that carried the tfdA gene. This method can be widely used to help resolve where in the community particular functional genes reside.

3. T-RFLP analysis of soil communities

3.1. Rationale for the method

The application of molecular techniques to microbial systematics has revolutionized our view of the phylogenetic relationships among bacteria (Woese, 1987; Olsen et al., 1994). The identification of ribosomal RNA as a premier molecule for evaluating evolutionary relationships has provided microbiologists with a much needed phylogenetic scaffold onto which new isolates can be placed and with which rational experiments can be designed. Indeed the subsequent application of this phylogenetic knowledge to microbial ecology has contributed enormously to our appreciation of microbial diversity. Perhaps the single most important facet of this knowledge has been the development of culture-independent approaches to the characterization of microbial communities. As evidence of this, the number of phylogenetic divisions has increased from the twelve, described by Woese based on cultivated organisms (Woese, 1987), to over 36 based on both, culture-dependent and independent approaches (Hugenholtz et al., 1998).

Prior to the phylogenetic revolution and the development of culture-independent molecular approaches,

estimations of diversity and evenness of microbial communities could not be made (Amann et al., 1995). A full description of community structure includes the number of species present (diversity), the number of individual cells of each species (evenness) as well as the physiological role of each species in the environment and its interaction(s) with other species. We now know that in many communities, including soil as mentioned above, the diversity is very high. However, the culture-independent approaches that have provided us with this insight, while definitive, are quite laborious (Pace et al., 1986). In order to identify biodiversity and species relatedness, the sequence of the 16S rRNA gene (or comparable phylogenetic marker) is needed. Hence, to assess the diversity in a complex environment, hundreds, perhaps thousands, of clones are required for comparative sequence analysis. While sequencing has become increasingly routine, it remains a laborious approach to comprehensively survey a microbial community.

As a result, other techniques have been developed for community analysis including *D*enaturing and *T*hermal *G*radient *G*el *E*lectrophoresis (DGGE/TGGE), *S*ingle *S*trand *C*onformation *P*olymorphism (SSCP), *R*estriction *F*ragment *L*ength *P*olymorphism (RFLP) or *A*mplified *R*ibosomal *D*NA *R*estriction *A*nalysis (ARDRA) and more recently *T*erminal *R*estriction *F*ragment *L*ength *P*olymorphism (T-RFLP). All of these techniques take advantage of 16S rRNA and culture-independent approaches. The initial steps include the extraction of community DNA followed by the PCR amplification of the 16S rRNA genes from the community DNA using universal, domain or group specific primers. The resulting products are separated in different ways, depending on the technique. In the case of DGGE/TGGE, the separation of PCR products that differ in sequence is based on relative helix stabilities in a denaturant or thermal gradient gel (Muyzer et al., 1993). This technique is very sensitive and has been used to detect single base differences. However, the gel system employed has low resolving power and, more importantly, there is no way of defining with accuracy the Tm of the helix. Moreover, there is no comparative sequence database that would provide insight into the relationship of Tm values to sequence. SSCP separates the PCR amplification products based on differences in electrophoretic mobility caused by conformational differences of folded single-stranded products (Lee et al., 1996). The conformational differences are, in turn, the result of differences in the primary sequence. SSCP has the same shortcomings as DGGE/TGGE mentioned above with the additional caveat that some single-stranded nucleic acids can exist in several metastable electrophoretically resolvable conformations. Thus, the assignment of an apparently unique conformer to a population or 'ribotype' is made with an element of faith.

Analysis with RFLP/ARDRA is based on the 16S rDNA-fragment length polymorphisms of restriction digests. Traditionally, the fragment lengths are determined with agarose or non-denaturing acrylamide gel electrophoresis. In general, this approach has been used most frequently on isolates as part of a clone screening step (Pace et al., 1986) prior to sequencing or, in some cases, to provide a level of insight into phylogeny (Moyer et al., 1996). More recently, the technique has been used to probe community structure (Massol-Deya et al., 1995; Smit et al., 1997). In these cases, it appears useful as a means to detect changes in communities, but has little utility in quantitating diversity or following specific ribotypes. This is especially true in complex communities where a single species can contribute four-to-six restriction fragments to the community pattern. In a complex community with many different species it quickly becomes apparent that a RFLP/ARDRA profile is too complex and, consequently, loses the phylogenetic information that is important in community analysis.

T-RFLP avoids many of the above-mentioned liabilities and yields community profiles that are complex but interpretable within the phylogenetic construct (Liu et al., 1997). Restriction fragment lengths can be determined for the entire ribosomal database and, therefore, provide a logical phylogenetic starting point. Experimentally, restriction fragment lengths can be determined down to ± 1.5 bases with sequencing gel technology. Moreover, by measuring only the terminal restriction fragment of each 16S rRNA gene, the complexity of the RFLP pattern is reduced and every visible band (fragment) is representative of a single ribotype or operational taxonomic unit. This, in turn, provides a quantitative basis for estimating diversity that, albeit imperfect, is more sensitive for quantitative measurements than the above-mentioned techniques.

3.2. Basic methodology

The initial description of the technique was presented by Liu et al. (1997). The protocol for analysis of a microbial community with T-RFLP is as follows. The initial steps for the culture-independent analyses are shared by all of the approaches and include the extraction of community DNA from an environment followed by PCR amplification of the 16S rRNA genes using primers constructed with the benefit of a 3000–5000 sequence database. The primers can be designed with domain level and, in some cases, division or group-level specificity. T-RFLP employs fluorescently tagged primers that can be detected by an ABI automated sequencer. Since the label is at the 5′ terminus, only the terminal fragment of a restriction digest is detected by the sequencer. After PCR amplification, the product is purified and, in individual reactions, digested with 2–4 restriction enzymes. The enzymes, which have given us the greatest resolution based on restriction analysis of the database as well as natural communities, are *Hha*I, *Rsa*I, and *Msp*I, but others may be of use under special circumstances. The digest is loaded onto an ABI sequencer and run on scan mode with internal size standards included in each lane. The output of the machine includes peak (fragment) height, area and fragment size in graphical and tabulated forms. The rate-limiting step in the protocol is the extraction and purification of community DNA of uniform quality. As the ABI sequencer can discriminate between different fluorescent tags in a single gel lane, a gel can be double and triple loaded by employing differently tagged primers in the PCR amplification step. This improves the cost effectiveness of the technique accordingly.

3.3. Results

As the terminal restriction fragment pattern for the entire database can be ascertained, any terminal fragment detected in a community can be compared with the database. In reality, members of closely related phylogenetic groups often share the same terminal restriction fragment size. Nevertheless, by employing 2–4 restriction enzymes, the combined effect is one of increasing the confidence in any phylogenetic inferences made. We have applied this technique to a variety of soil types and other microbial communities

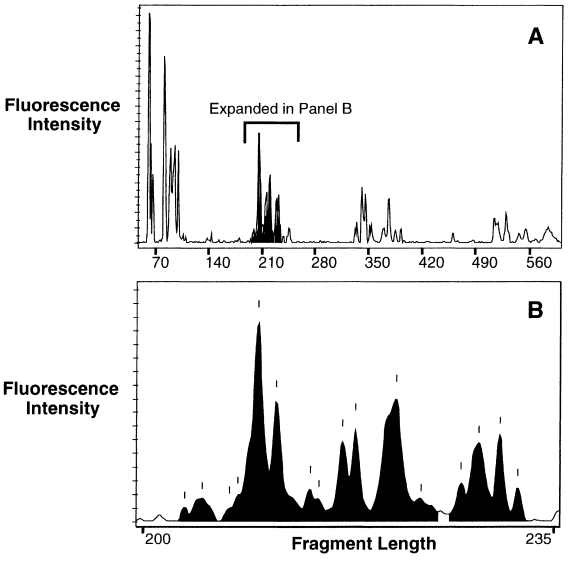

Fig. 3. T-RFLP of a soil bacterial community. Panel A. Electropherogram of an *Hha*I digest of PCR-amplified 16S rRNA genes. The fluorescent label was at the 5′ terminus of the 8F eubacterial-specific primer. The PCR products were purified, digested with *Hha*I, and loaded onto an ABI automated sequencer. The data presented were analyzed with ABI GeneScan 2.1 software. The range is expanded in panel B to demonstrate the resolution power of the sequencing gel system. This small region of the gel can resolve at least 16 terminal restriction fragments.

with considerable success. In every case, our ability to detect and track populations (ribotypes) is at least five times greater than that using DGGE. Fig. 3(A) presents a T-RFLP analysis of a soil community using eubacterial specific primers. The fragment size range is from 50 to 600 bases. Fig. 3(B) presents an expanded size range with individual peaks (fragments) identified with a hash mark. In this particular soil, 75 terminal fragment sizes could be detected between 50 and 600 bases. When a group-specific primer (*Cytophaga*) was used on the same community, the number of detectable terminal fragments was reduced to ten (data not shown), the sizes of which were consistent with predictions based on the 16S ribosomal RNA database.

The technical nature of T-RFLP is such that a high throughput of samples is possible. This, in turn, permits microbial ecologists to take advantage of rigorous comparative community analysis that includes population demographics as well as process analysis. For example, we examined three other sig-

nificantly different soil samples, based on geochemical measurements, from the same general site as the sample presented in Fig. 3. We were able to identify up to 34 ribotypes that are common to the four samples as well as 8–15 that are unique to certain soils. The ability to discriminate ribotypes at this level will help us to dissect microbial communities phylogenetically, align physiological abilities with taxonomic units and gain greater understanding of population interactions within a community. T-RFLP can be coupled with other techniques, such as the G + C analysis described above, as well as with enrichment protocols and other culture-dependent approaches. As an adjunct to T-RFLP, a supporting web site that integrates the Ribosomal Database Project with terminal restriction fragments is under construction at the Center for Microbial Ecology/Michigan State University (http://www.cme.msu.edu)

4. ARDRA analysis of soil communities and of isolates

4.1. Rationale for the method

Just as the T-RFLP method, ARDRA is based on PCR amplification of the 16S rRNA genes present in soil DNA. ARDRA, however, provides a higher level of resolution than T-RFLP because the sizes of all restriction fragments are used in the analysis for each enzyme, therefore sampling a larger portion of the *rrn* sequence. A computer analysis of digestion sites of over 100 environmental strains in the ribosomal database showed that the median sequence difference detected by the use of four tetrameric restriction endonucleases was 97.4% (Moyer et al., 1996). Since organisms that are <97.5% similar in 16S rRNA sequence are not known to be of the same species (Stackebrandt and Goebel, 1994), this indicates that the 16S rRNA gene resolved by four enzymes are at least from different species. The 16S rRNA clones differentiated by restriction enzymes are often termed OTUs or phylotypes.

The ARDRA technique can be used on the complex population of rRNA PCR products directly amplified from community DNA, from clone libraries in which these PCR products are maintained in *E. coli*, or in PCR products generated from pure cultures.

The first approach is sometimes termed 'community ARDRA', since the bands on the gels should reflect the population of all restriction fragments for at least the major members of the community which yielded amplicons (Massol-Deya et al., 1995). As mentioned in Section 3, the community ARDRA approach does not work well in soils with highly diverse and non-dominant populations since too many bands are produced to be resolved. It does, however, work if the community is enriched for a few dominant members, such as by the addition of a single substrate (Fries et al., 1997, and below). In this case, the succession of dominant community members can be followed. In addition, the ARDRA patterns of isolates and of rDNA clones can be compared with those from community DNA to see if they are the same and, hence, suggest the identity of the dominant members of the community.

The second use of this approach is with clone libraries (in *E. coli*) of 16S rRNA genes; this has now been done with DNA from a number of soils (Liesack and Stackebrandt, 1992; Ueda et al., 1995; Borneman et al., 1996; Borneman and Triplett, 1997: Kruske et al., 1997; Zhou et al., 1997; Felske et al., 1998; Nüsslein and Tiedje, 1998). In this application, the community of PCR amplicons are cloned into *E. coli* cells to separate the PCR products into individual cultures so that each 16S rDNA clone can be analyzed separately. This method provides better resolution of the community but is very time-consuming, since a large number of clones must be analyzed individually. This is the major reason why the T-RFLP method has become so popular. With a large number of clones, the efficient screening of those clones is crucial. Currently, the more rapid way to screen such a clone library for distinguishing different clone types is by restriction analysis, i.e. ARDRA. Results of such an analysis is shown in Fig. 2(b). Since each clone can be cultured separately, the entire gene can also be readily sequenced for further resolution of differences as well as to compare the sequence with a database of ribosomal sequences, RDP (http://www.cme.msu.edu/RDP). This method has revealed a very large number of very novel rRNA genes in soils (Borneman et al., 1996; Borneman and Triplett, 1997: Kruske et al., 1997), some of which represent very early branches in evolution. Some of these newly discovered types have no cultured representatives, so their physiology and

role in the soil community is unknown. These methods, however, have shed the first light on their existence in soil.

The use of ARDRA is illustrated below in a study in which we analyzed the development of 2,4-D degrading populations in adjacent soils with three different life histories:

(i) continuously cultivated agriculture;
(ii) no-till agriculture; and
(iii) never tilled, successional old fields.

4.2. Effect of 2,4-D on the microbial community as influenced by soil management history

For the purpose of investigating the effect of management history of soil on the soil bacterial communities, soil microcosms were constructed from conventional till, no-till, and successional old field soils. The objective of the study was to compare the responses of three soil microbial communities to applications of the model herbicide 2,4-D. It was hypothesized that these different land-use practices and differences in prior exposure to 2,4-D will result in significant differences in the structures of the resident communities and that their responses to the experimental application of 2,4-D would be different. One would also expect that the resident 2,4-D degrading species would be different in the three soils.

Soils were collected from the long-term ecological research (LTER) site at the W.K. Kellogg Biological Station in southwest Michigan (http://lter.kbs.msu). Three of the eight long-term treatments established in 1989 were sampled for this study. These were: Treatment 1 (conventional tilled soil) in which the soil had been under cultivation for about 40 years prior to 1989 at which time the soil was placed under a high input corn, soybean rotation as well as conventionally tilled; Treatment 2 (zero-tilled soil) has the same history as Treatment 1, but in 1989 it was placed under continuous no-till management. Both, treatments 1 and 2 had 2,4-D applications as part of their agricultural history. Treatment 8 (successional field) was an unfertilized successional field left to regrowth of native plant species for the past 40 years. It has never been tilled and no 2,4-D has been applied.

Soil samples were collected with a 2.5-cm diameter soil probe and 10 soil subsamples were taken randomly from each plot, composited, and sieved through a 2-mm pore sieve. Microcosm studies were initiated with the soil samples and incubated at 25°C in the dark. 2,4-D at 100 ppm dissolved in 0.1 M phosphate buffer (pH 7.0) or phosphate buffer alone was added to 300 g of soil in a polyethelene bag, such that each microcosm received identical concentrations of phosphate and either 0 or 100 ppm 2,4-D. The moisture content was adjusted to 25% (wt/wt) with sterile distilled water. 2,4-D concentration in the soil was monitored by HPLC and 2,4-D was reapplied whenever the concentration dropped below 10 ppm. Soil samples were taken to extract total bacterial DNA before 2,4-D treatment and after the fifth application of 2,4-D had been consumed. Total 2,4-D degraders were estimated by the most probable number (MPN) procedure, and the total heterotrophic count was determined before, and after, 2,4-D application. Thereafter, 16S rRNA gene analysis of the dominant population was determined by T-RFLP and community ARDRA.

2,4-D degraders were isolated from the highest MPN dilution that exhibited 2,4-D degradation after enrichment by two additional transfers into fresh medium. Each enriched culture was streaked on 2,4-D minimal agar medium containing 300 ppm 2,4-D, 0.1% casamino acids, and 1.5% agar and incubated at 30°C for 2–7 days. Some of the colonies were tested for 2,4-D degradation and in fresh 2,4-D liquid medium. The purity of the isolates was confirmed by streaking on R2A medium. The isolates were analyzed to determine whether they were closely related by *rep*-PCR (Versalovic et al., 1994). They were analyzed phylogenetically by ARDRA. The 16S rRNA gene was partially sequenced for selected isolates with different ARDRA patterns and their evolutionary relatedness to known organisms established.

4.3. Results

The MPN count of 2,4-D degraders was initially very low (21 2,4-D degraders/g) in the non-agricultural treatment, presumably because this pristine environment had no history of 2,4-D application, compared with 4.3×10^4 and 1.4×10^4 2,4-D degraders/g in treatments 1 and 2, respectively. The initial rate of 2,4-D degradation was also low in the non-agricultural soil, but the rate eventually became

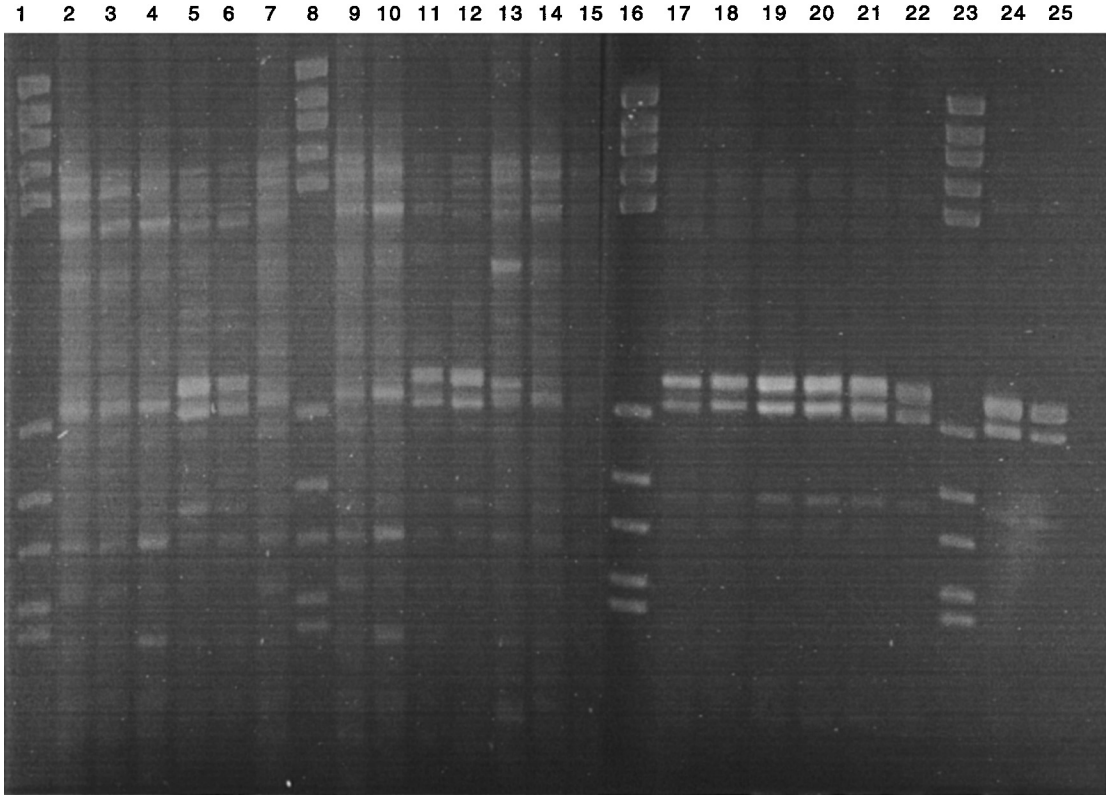

Fig. 4. Effect of soil management history on dominant 2,4-D degraders as revealed by ARDRA analysis of 16S rDNA PCR products amplified directly from the soil DNA (left side) and ARDRA analysis of the 16S rDNA genes of isolates from these communities (right side). Treatment 1, lanes 2–4 before 2,4-D application and lanes 5–6 after 2,4-D application. Treatment 2, lanes 7, 9, 10 before 2,4-D application and lanes 11–12 after application. Treatment 8, lanes 13–15 before 2,4-D application and lanes 17–18 after 2,4-D application. Lanes 19—25 are of isolates from all three treatments. Lanes 1, 8, 16, 23 are size markers.

equivalent to that of the other treatments after additional 2,4-D amendments. All three sites had populations in the range of 0.5×10^9 to 3×10^9 2,4-D degraders/g after the five additions of 2,4-D. The community ARDRA pattern of pre- and post-2,4-D application demonstrated a shift in the microbial community in all treatments after the 2,4-D addition (Fig. 4). The community ARDRA profiles were similar for all three soils suggesting that the dominant members were either the same or closely related in soils with the three different life histories, rejecting the hypothesis that life history would cause different 2,4-D degrader community structures. The T-RFLP analysis demonstrated a similar shift between pre- and post-treatment communities, namely a shift in bands in the 400 and 500 bp region to bands in the 50–150 bp region (data not shown). In contrast, treatments 1 and 2, but not Treatment 8, showed a 700 bp band before 2,4-D application, but after 2,4-D application that band disappeared.

2,4-D degraders were isolated from all three treatments and characterized by ARDRA (Fig. 5). These gel patterns have been digitized and the strains grouped by a clustering program (GelCompar, Applied Maths, Kortrijk, Belgium). The dendrogram produced by this program shows the degree of similarity in the ARDRA patterns and indicates that the strains from the site fall into three clusters (Fig. 5). The cluster comprised the strains at the right of the figure, strains 2, 15, 7, 9, 20, 21, 18, and 19, have ARDRA patterns that match the community ARDRA amplified directly from soil (Fig. 4 right side (isolates) compared with Fig. 4 left side (soil)), suggesting that these isolates were the dominant strains in the

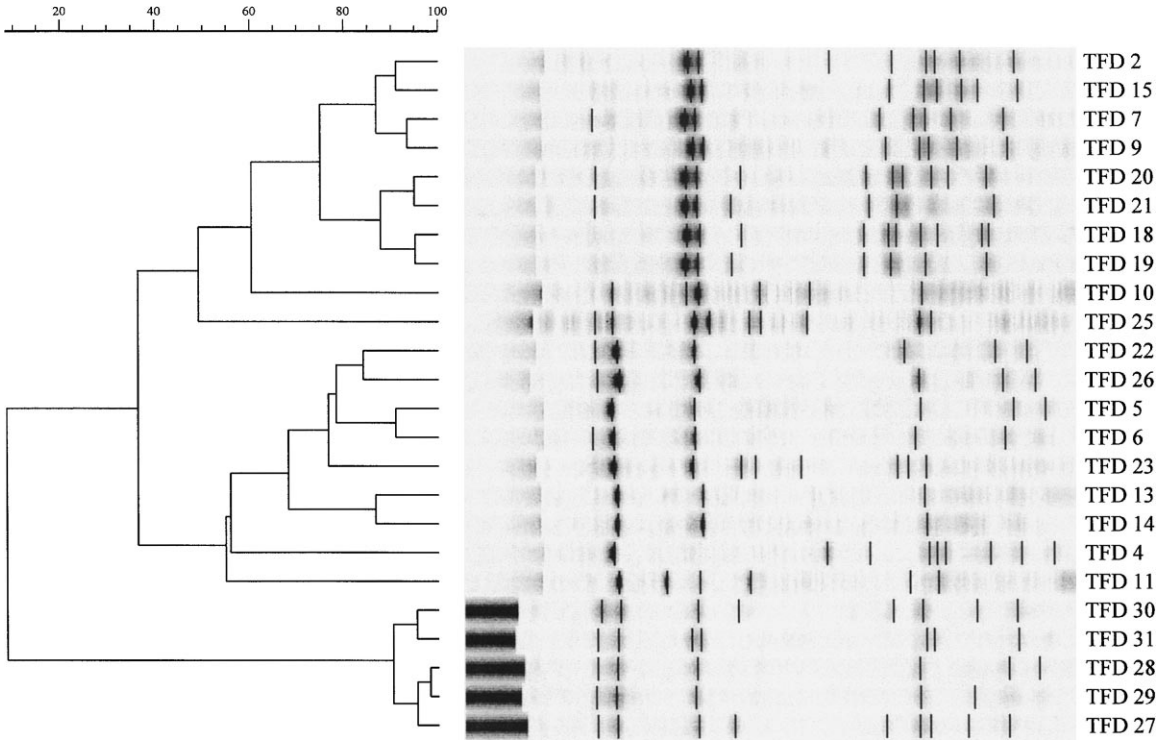

Fig. 5. Results of cluster analysis of ARDRA patterns of a collection of 2,4-D degrading isolates from the LTER site. Isolates TFD 25–30 were collected over five years ago from a different location on this site (Ka et al., 1994). The rest of the isolates are from the current study. Three phylogenetic groups of strains are apparent.

soil that responded to the 2,4-D treatment. Partial sequencing of the 16S rRNA gene from some of these isolates revealed that they belong to the *Burkholderia* genus. Members of this genus are important soil organisms, including rhizosphere colonizers, and are common degraders of 2,4-D and other manufactured chemicals.

The results obtained so far indicate that the management history of the soil did not affect the bacterial communities selected by 2,4-D addition. Since the dominant members observed by community and isolate ARDRA analysis gave the same OTU pattern, these profiles could be from identical organisms, but if not, they are at least closely related. The ARDRA method, which provides a moderate level of resolution, will therefore not resolve strains that are closely related but can still have significant differences in their phenotype. Cloning and sequencing of the dominant bands will elucidate how nearly identical these strains might be.

This study demonstrates how community and isolate ARDRA patterns can be used to analyze succession and to identify dominant members of a community responding to particular treatments.

5. *rep*-PCR

Perhaps the most popular fine-scale method to resolve differences in populations between different sites is the use of *rep*-PCR (repetitive extragenic palindromic-PCR) (Versalovic et al., 1994). Genomic fingerprints of chromosome structure can easily be obtained by using primers that bind to randomly interspersed repetitive DNA sequences around the chromosome. Chromosome structure is considered to be rather variable among strains and, hence, the reason why this method provides a fine level of resolution of differences among strains. *rep*-PCR, however, requires the use of isolates. It is often used

as the first-level screen to indicate how closely related the isolates are. A protocol for use of the GelCompar program to analyze for relatedness in large strain collections has recently been described by Rademaker et al. (1998). This method can be used directly on small amounts of cells, e.g. a colony, without prior DNA extraction, thus making it possible to analyze 60–100 stains overnight. The method is rapid, reproducible, the data were suitable for storage in a searchable database, and provides the highest level of taxonomic resolution of any current PCR-based method (Vinuesa et al., 1998). There are three primer sets that have been found to work in most bacteria; these are known as REP, BOX, and ERIC. Of these, BOX has been found to be the most useful, because it works reliably on new strains and gives the maximum band patterns, hence a higher degree of resolution among strains. This method is most effectively used when it is targeted to populations selected to be of a certain phenotypic or taxonomic group. It has been successfully used to differentiate organisms like *Rhizobium* (Vinuesa et al., 1998), plant pathogenic *Xanthomonas* (Louws et al., 1994), fluorescent pseudomonads, and populations that grow on particular substrates. It is most effective in resolving organisms at the subspecies level of resolution.

We also have used the *rep*-PCR method to evaluate the degree of endemism in soil populations, i.e. how geographically unique are particular populations. This has important implications for determining where new microbial diversity resides and the extent of prokaryotic diversity on Earth. In one early study, a high degree of endemism was found in chloroaromatic-degrading populations from around the world (Fulthorpe et al., 1998).

6. Conclusions

The tools of molecular biology were first applied to soils a decade ago. Many obstacles made progress minimal in the first years. Particularly problematic was the difficulty in comprehensive recovery of DNA that was sufficiently pure for reliable PCR, cloning, hybridization, and restriction analysis. This problem has now largely been solved, although fine tuning of extraction and clean-up methods is often needed for each particular soil. Now, the rate of discovery of new knowledge using molecular techniques is growing rapidly and the rate is expected to continue to increase because of the further improvement of methods particularly tuned for environmental work and the extent of the unknown character of the soil community. Soil microbiology is truly the frontier of biology.

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