Microbial Ecology

Denaturing Gradient Gel Electrophoresis Can Rapidly Display the Bacterial Diversity Contained in 16S rDNA Clone Libraries

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Abstract

Two different strategies for molecular analysis of bacterial diversity, 16S rDNA cloning and denaturing gradient gel electrophoresis (DGGE), were combined into a single protocol that took advantage of the best attributes of each: the ability of cloning to package DNA sequence information and the ability of DGGE to display a community profile. In this combined protocol, polymerase chain reaction products from environmental DNA were cloned, and then DGGE was used to screen the clone libraries. Both individual clones and pools of randomly selected clones were analyzed by DGGE, and these migration patterns were compared to the conventional DGGE profile produced directly from environmental DNA. For two simple bacterial communities (biofilm from a humics-fed laboratory reactor and planktonic bacteria filtered from an urban freshwater pond), pools of 35–50 clones produced DGGE profiles that contained most of the bands visible in the conventional DGGE profiles, indicating that the clone pools were adequate for identifying the dominant genotypes. However, DGGE profiles of two different pools of 50 clones from a lawn soil clone library were distinctly different from each other and from the conventional DGGE profile, indicating that this small number of clones poorly represented the bacterial diversity in soil. Individual clones with the same apparent DGGE mobility as prominent bands in the humics reactor community profiles were sequenced from the clone plasmid DNA rather than from bands excised from the gel. Because a longer fragment was cloned (~1500 bp) than was actually analyzed in DGGE (~350 bp), far more sequence information was available using this approach that could have been recovered from an excised gel band. This clone/DGGE protocol permitted rapid analysis of the microbial diversity in the two moderately complex systems, but was limited in its ability to represent the diversity in the soil microbial community. Nonetheless, clone/DGGE is a promising strategy for fractionating diverse microbial communities into manageable subsets consisting of small pools of clones.

Introduction

A primary objective of microbial ecologists is to describe the microbial community diversity in environmental samples. Isolation of environmental bacteria on laboratory media allows elaborate physiological characterization of the isolates [22]. However, it is by now almost a cliché to acknowledge that culturing detects only a small fraction of the total number of species present, and that molecular methods targeting 16S rDNA more completely describe bacterial diversity [2]. In general, two divergent molecular approaches have been used for microbial community analysis. One approach has been to clone community 16S rDNA polymerase chain reaction (PCR) products, screen clone libraries with restriction enzymes, and sequence from one to several clones from each restriction pattern [14]. The clone/sequencing approach is time and labor intensive [9, 20], which has no doubt increased the popularity of molecular profiling techniques that give a "snapshot" of the entire community (reviewed by Tiedje *et al.* [27] and Torsvik *et al.* [28]). Of these molecular methods, denaturing gradient gel electrophoresis (DGGE) is probably the most popular because of its relatively high resolution and because DNA sequence information can be recovered from gel bands [19, 20, 33].

DGGE has disadvantages, however. Informative gels with well-resolved bands are difficult to produce, especially from samples that contain low biomass and/or are contaminated with humics or other compounds

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that inhibit PCR. Also, diverse microbial communities, such as soil, test the resolution limits of DGGE. DGGE images from soil very likely capture a mix of real diversity and artifact. In many instances, recovering DNA sequence information from excised gel bands ultimately requires cloning [3, 15]. Furthermore, the DNA sequence information available from DGGE is limited by the size of the PCR fragment amplified, usually <500 bp [6, 20].

Here we present evidence that cloning and DGGE can be combined into a single protocol (clone/DGGE) that takes advantage of the benefits of each: the ability of DGGE to display a profile of the whole bacterial community and the ability of cloning to package pure DNA sequence information. Environmental DNA was amplified using primers for 16S rDNA, and the PCR products were cloned. Individual clones were screened by analyzing their DGGE mobilities next to the conventional community profile that was produced from extracted DNA without a cloning step (direct DGGE). In addition, pseudocommunity profiles were constructed by pooling bacterial cells from 35 to 50 individual clones, purifying their plasmid DNA, and performing DGGE. The similarities between the pooled clone/DGGE and direct DGGE profiles gave a visual estimate of the coverage of the library that was achieved by the number of clones pooled. Clones comigrating with intense bands in the profiles were sequenced from plasmid DNA. By cloning near full-length 16S rDNA fragments, we could sequence a longer fragment than was actually analyzed by DGGE.

We tested the concept of clone/DGGE using DNA extracted from three different environments with a range of expected complexity: a laboratory biofilm reactor that was fed with humics as substrate, a freshwater pond, and a soil. The purpose was not to compare the diversity of any of these samples, but rather to show how the diversity of any single environment could be displayed. Although Muyzer and Smalla [20] noted that DGGE could be used to screen clones, to date, few researchers have used DGGE in this way. Liu *et al.* [17] amplified the entire cloned insert with primers targeting the vector (a screen for correct insert size) before using a nested PCR for DGGE. Avrhami and Conrad [3] analyzed individual clones and the whole community (direct DGGE) in the same gel. Webster *et al.* [30] used DGGE to estimate the microbial diversity prior to cloning. However, we believe that we are the first to demonstrate the full potential of combining cloning and DGGE for the analysis of microbial communities.

Methods

Figure 1 is a flowchart illustrating strategies used in this study to analyze the microbial communities from different environments. Either partial or near full-length PCR products were cloned (solid arrows). Individual clones or pools of clones were then screened using DGGE. Migrations of individual clones or the patterns from the pooled clones were compared with direct DGGE profiles, which were produced either from the original DNA extract or from existing PCR products (dashed arrows). In either case, they were considered "direct" in the sense that no cloning was involved. Samples for this research included biofilm grown in a laboratory reactor, water from an urban freshwater pond, and surface soil from an urban lawn. Each was a source of one sample of DNA for analysis.

Biofilm Reactor. Biofilm was grown on polycarbonate coupons in a rotating disk reactor (BioSurface Technologies, http://www.biofilms.biz) that had a liquid volume of approximately 450 mL. A concentrated humics solution was prepared by dissolving 0.96 g of Suwanee River natural organic matter (International Humic Substances Society, http://www.ihss.gatech.edu) in 500 mL of autoclaved deionized water. The solution was mixed on a stirplate for 24 h, after which the total organic carbon content was measured at 760 mg C L^{-1} on a Shimadzu TOC-5000A Total Organic Carbon Analyzer (http://www.ssi.shimadzu.com). This stock solution was stored at 4°C, and an aliquot was added to 20 L of autoclaved deionized water in a glass carboy (that had been baked at 500°C for 8 h) to make a feedstock of 10 mg C L^{-1} (~90 μM C). A second baked 20-L glass carboy was filled

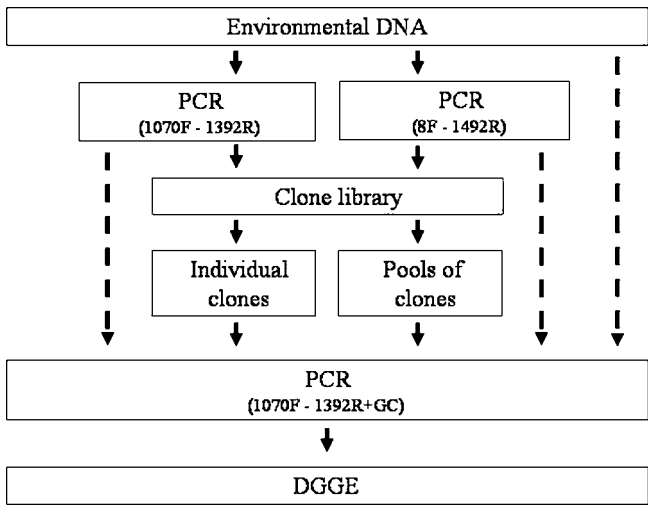

Figure 1. Flow diagram illustrating protocols used in this study. Conventional or direct DGGE did not involve a cloning step (*dashed arrows*). Templates for direct DGGE were either extracted DNA or existing PCR products. Either a partial or near full-length PCR fragment was cloned. The clone library was screened using DGGE. Either individual clones or pools of clones were analyzed. Clone pool and direct DGGE profiles were compared, and individual clones were identified that comigrated with specific bands in the profiles.

with a sterile solution that had a nitrogen concentration of 9 μM from KNO_3 and a phosphorus concentration of 0.9 μM from $NaH_2PO_4{\cdot}2H_2O$. Approximately 18 mL h^{-1} from each of the two feed carboys was metered into the biofilm reactor along with dilution water for a total flow rate of 180 mL h^{-1}, a final C concentration of ~1 mg C L^{-1}, and a molar C/N/P ratio of 100:10:1. Dilution water for the reactor was municipal tap water that had been dechlorinated by running it through a biologically active carbon column that was also a source of inoculum for biofilm growth. The 2.5-h hydraulic residence time in the reactor was considered too short to support growth of planktonic cells. The humics reactor was operated for 6 weeks.

Sampling and DNA Extraction. Biofilm was sampled from the humics reactor after 6 weeks by aseptically scraping material from coupons into 10 mL of sterile water, followed by serial centrifugations to pellet the cells in a 2-mL tube for subsequent DNA extraction. For the pond water sample, approximately 200 mL was filtered through a 0.2-μm pore size polycarbonate filter (25-mm diameter). The filter was then aseptically shredded with forceps and placed into a 2-mL tube. Surface soil was sampled aseptically from just below the duff layer of an urban lawn and well mixed, and approximately 0.5 g (fresh weight) was placed in a 2-mL tube. DNA was purified from these 2-mL sample tubes using the Bio101® Fast DNA® Spin® Kit for Soil (http://www.qbiogene.com) following the manufacturer's protocol except for the following modification to remove humics. Once the DNA had been bound to the suspended proprietary silica matrix, this matrix was washed in 500 μL 5.5 M guanidine thiocyanate and pelleted, and the supernatant was discarded. This procedure was repeated until the brown color associated with humics had disappeared. The manufacturer's protocol was then resumed to completion. DNA yield was estimated on an agarose gel with ethidium bromide staining, serial dilutions were performed, and the DNA preparations were stored at −20°C.

PCR and Cloning. Oligonucleotide primers (Table 1) were synthesized by Integrated DNA Technologies (http://www.idtdna.com). Primers 8F and 1492R were used to produce near full-length (~1500 bp) 16S rDNA amplicons (humics reactor and soil DNA). Shorter products (~350 bp) were amplified using primers 1070F and 1392R (freshwater pond DNA). Primers VectF and VectR targeted sequences immediately flanking the vector cloning site and were used to screen clones for correct insert size. Primer 1392R was also synthesized with a 5′ 40-bp GC clamp [8] and was paired with primer 1070F for amplifying fragments to be analyzed by DGGE. PCR reactions (20 or 50 μL) were performed using 2X PCR Master Mix (http://www.promega.com). The PCR reaction mixture consisted of 1.5 mM $MgCl_2$, 200 μM each dNTP, 0.5 μM forward and reverse primers, 0.025 U μL^{-1} *Taq* DNA polymerase, and 1–5 μL template (approximately 20–200 ng DNA) in a proprietary buffer (pH 8.5). All PCR amplifications were performed on a PTC 100 thermal cycler (MJ Research, Inc., http://www.mjr.com) using a touchdown program. An initial denaturation for 5 min at 94°C was followed by a total of 25 cycles of amplification consisting of (1) denaturation at 94°C for 45 s and (2) touchdown annealing (5 cycles at 60°C, 5 cycles at 58°C, 5 cycles at 56°C, 10 cycles at 55°C) for 45 s, and extension at 72°C for 90 s. The program ended with an extension step at 72°C for 5 min. PCR products were confirmed by agarose gel electrophoresis and staining with ethidium bromide and used for either cloning or were analyzed by DGGE.

PCR products were cloned into plasmid vector pCR 2.1 using the TOPO TA Cloning kit (Invitrogen, http://www.invitrogen.com) following the manufacturer's protocol. White colonies were used to inoculate sterile microcentrifuge tubes containing 0.5 mL of Luria-Bertani (LB) broth plus 50 μg mL^{-1} ampicillin or kanamycin, which were incubated overnight at 35°C in a shaking incubator. Pools of clones were obtained by pipetting 20-μL stationary phase LB broth from 35 to 50 clones into a single 2-mL tube. Plasmid DNA was purified from the pools or from individual clones using the Wizard Plus SV Minipreps kit (http://www.promega.com) and stored at −20°C. Plasmid DNA was amplified for DGGE analysis using the same PCR reagents and conditions.

Table 1. Oligonucleotide primers used in this study

Identity	*Sequence (5′ to 3′)*	*Specificity*	*Reference*
8F	AGAGTTTGATCCTGGCTCAG	Bacteria	[2]
1070F	ATGGCTGTCGTCAGCT	Bacteria	[8]
1392R	ACGGGCGGTGTGTAC	Universal	[8]
1492R	GGTTACCTTGTTACGACTT	Bacteria	[17]
VectF	AGTGTGCTGGAATTCGCC	Vector pCR 2.1	This study
VectR	ATATCTGCAGAATTCGCC	Vector pCR 2.1	This study
GC clamp	CGCCCGCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCC		[8]

DGGE and DNA Sequencing. Denaturing gradient gel electrophoresis was performed on PCR products from isolates, from community chromosomal DNA, from individual clone plasmid DNA, or from plasmid DNA from pools of clones, using a DCode™ system (http://www.biorad.com) and reagents from Sigma-Aldrich (http://www.sigmaaldrich.com). Denaturation conditions were optimized based on preliminary results, but generally, gels had denaturation gradients of either 50–70 or 40–60%, where 100% denaturant is defined as 7 M urea and 40% formamide. Gels were either 8% polyacrylamide or contained an 8–12% polyacrylamide gradient [11]. Electrophoresis was at 60 V for 16 h. Gels were stained with Sybr®Gold (http://www.molecularprobes.com) and documented using a FluorChem™ 8800 fluorescence imager (http://www.alphainnotech.com). Bands in DGGE images were identified visually on a presence–absence basis. Band intensities were not physically measured, but visually prominent bands were identified to be matched with individual clones. Clones were sequenced in each direction from purified plasmid DNA with either primer 1070F or 1392R using the Big Dye™ Terminator Cycle Sequencing kit (http://www.appliedbiosystems.com). Sequencing reaction products were analyzed on an ABI310 DNA sequencer (http://www.appliedbiosystems.com).

Results

Denaturing gradient gel electrophoresis was used to screen 16S rDNA clone libraries from three different environments that differed in their expected level of microbial diversity, including a biofilm reactor, a freshwater pond, and soil from a lawn. The microbial community in the biofilm reactor was expected to be the least diverse because the experimental design selected for bacteria was able to use humics as substrate and to exist in a biofilm. This community was analyzed in several steps. First, near full-length PCR products from biofilm DNA were cloned (primers 8F and 1492R). Plasmid DNA was purified from 10 clones and used as template in a nested PCR to compare the migration of these clones with dominant bands in a DGGE profile that was produced directly from biofilm DNA (primers 1070F and 1392R + GC, results not shown). This preliminary image indicated two dominant bands (one very intense, one less intense) and another 8–10 bands that could be distinguished from the background. It also identified candidate clones that appeared to align with the two dominant bands. Next, a pseudocommunity was reconstructed by pooling 20 μL of stationary phase LB broth cultures from each of 50 clones randomly selected from the library and purifying the plasmid DNA from this clone pool. Finally, a single image was created that combined the direct community DGGE profile, the DGGE profile of the 50 pooled clones, and individual clones that matched the migration of the two most dominant bands (Fig. 2). A single band obtained from the *Escherichia coli* cloning host, included in this analysis as a control (lane 6), had a different DGGE migration from any of the 10 clones screened and did not appear in the profile of the pooled clones, eliminating cloning host contamination as a concern in these plasmid preparations.

The similarity in the direct and pooled DGGE profiles suggests that 50 random pooled clones contained most of the dominant genotypes present in the whole community and in roughly the same proportions. Major arrows indicate bands apparently common to both profiles, but at least three bands (minor arrows) in the direct DGGE were not matched by clones in the pool. Partial 16S rDNA sequence (primers 1070F and 1392R) was obtained from the three selected clones using individual plasmid preparations rather than excised gel bands. BLAST searches [1] of GenBank (http://www.ncbi.nih.gov) indicated that the two clones (lanes 2 and 3) that comigrated with the most intense band in Fig. 2 were 100% identical to a sequence recovered from a drinking water distribution system biofilm (accession no. AY328738, [31]), and that one clone that comigrated with the second dominant band (lane 1) was 100%

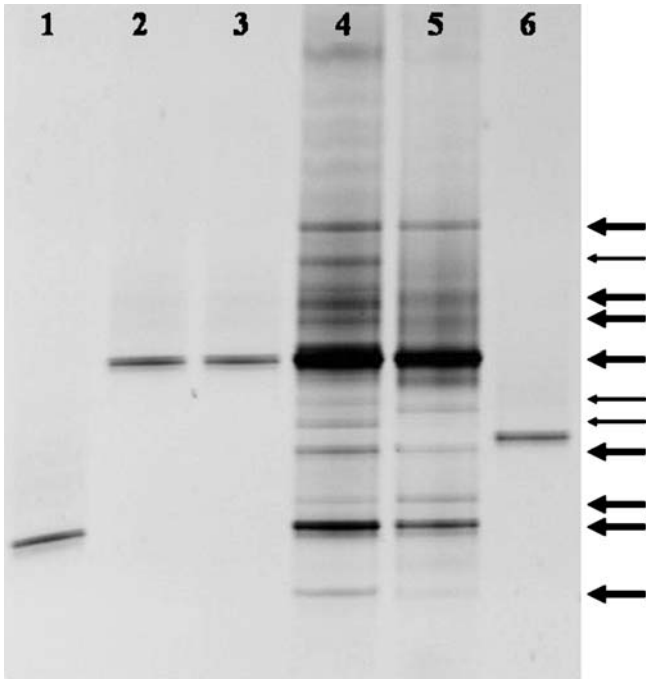

Figure 2. DGGE analysis of a biofilm from a humics-fed laboratory reactor. *Lanes 1–3*, individual clones. *Lane 4*, the direct DGGE profile. *Lane 5*, the DGGE profile produced from a pool of 50 clones. *Lane 6*, the band from the *E. coli* cloning host. *Major arrows* indicate bands that appear to be shared by both profiles. *Minor arrows* indicate bands in the direct DGGE profile that are not matched by bands in clone pool DGGE profile.

identical to a sequence identified in humics studies of lakes and mesocosms (accession no. AJ629862; Haukka *et al.*, unpublished data).

The same approach was used to analyze the microbial community diversity in a freshwater pond, except that only partial 16S rDNA products (primers 1070F and 1392R) were cloned. A preliminary direct DGGE profile (primers 1070F and 1392R + GC) gave an estimate of the diversity present and was also used to screen 12 individual clones (results not shown). The direct DGGE profile, the DGGE profiles of two separate pools of 35 clones, and the migrations of several selected clones are shown in Fig. 3. As was the case with the humics reactor, the freshwater pond was apparently dominated by a few genotypes. Two dominant bands are common to all three profiles and appear to match the migrations of two individual clones (lanes 5 and 6), making these clones candidates for DNA sequencing. One clone pool (lane 2) appears to contain clones matching the migration of at least seven bands (major arrows) visually identified in the direct DGGE profile (lane 1). One band in the direct DGGE profile (minor arrow) does not appear to have a match in either clone pool. Each clone pool contains some bands not present in the other pool or in the direct DGGE profile.

Finally, the feasibility of using DGGE to screen clones from a complex microbial community was tested. DNA from a lawn soil was used as template to amplify near full-length 16S rDNA products (primers 8F and 1492R), which were then cloned. A library of 100 clones was produced, and plasmid DNA from two separate pools of 50 clones was purified, with replication. Figure 4 compares the direct DGGE profile to the profiles of the two clone pools (primers 1070F and 1392R + GC). In this case, the template for the direct DGGE profile was the same near full-length PCR product that was cloned, rather than the original DNA extract. The similarity between replicates from the same clone pool indicates reproducibility in the pooling, plasmid purification, PCR, and DGGE. However, the remarkable differences between the two pools and the large number of bands in the direct DGGE profile that are not represented in either pool are evidence that far more than 50–100 clones should be screened to analyze the microbial diversity in this sample. Furthermore, no more than 30 distinct bands could be counted in either clone pool profile, suggesting comigration of some clone bands. Despite the overall inadequate representation of the direct DGGE profile by the clone pools, some band locations in the direct DGGE profile are matched by bands in the clone pools (e.g., bands indicated by major arrows). Other prominent bands in the direct DGGE profile have no match in the clone pool profiles (e.g., bands indicated by minor arrows). As always, comigration of bands does not indicate the same genotype. The direct DGGE profile (lane 5) is typical of patterns from soil, suggesting a high degree of both richness and evenness. However, the mix

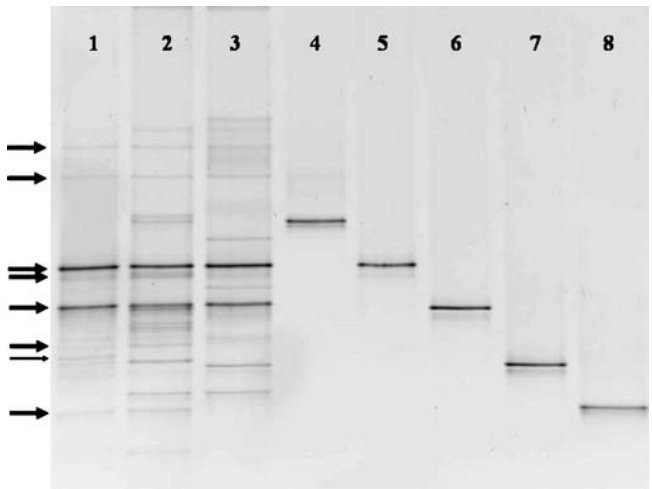

Figure 3. DGGE analysis of a freshwater pond microbial community. *Lane 1*, the direct DGGE profile. *Lanes 2 and 3*, the DGGE profiles produced from two separate pools of 35 clones. *Lanes 4–8*, individual clones. *Major arrows* indicate bands shared by the direct DGGE and first clone pool DGGE profile (*lane 1* vs *lane 2*). The *minor arrow* indicates a band in the direct DGGE profile that is not present in the first clone pool DGGE profile.

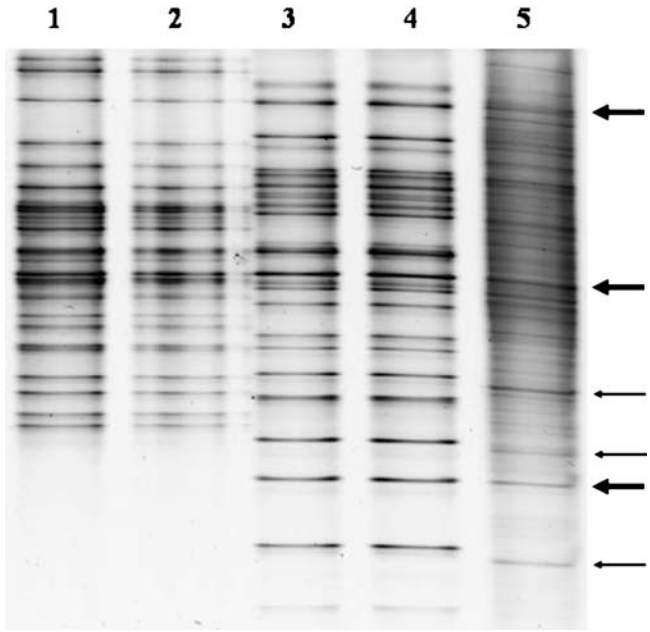

Figure 4. DGGE analysis of a lawn soil microbial community. *Lanes 1 and 2*, DGGE profiles of replicate pools from clones 1 to 50. *Lanes 3 and 4*, DGGE profiles of replicate pools from clones 51 to 100. *Lane 5*, the direct DGGE profile. *Major arrows* indicate examples of bands apparently common to the direct DGGE profile and at least one of the clone pools. *Minor arrows* indicate bands in the direct DGGE profile apparently not matched by clones in either pool.

of intense and weak bands close together is superimposed on a background that could consist of either additional diversity or artifact, making it nearly impossible to determine the total number of bands, either visually or by gel imaging software.

Discussion

Neither DGGE nor cloning/sequencing is entirely satisfactory for analysis of microbial communities. Cloning/sequencing is laborious, and unless clones are screened, it often results in redundant sequencing. For example, McGarvey *et al.* [18] sequenced 500 clones, without screening, from each of three libraries from a dairy wastewater environment, but identified only about 150 different operational taxonomic units per library. On the other hand, in several typical studies using DGGE, cloning was ultimately unavoidable; PCR products from excised bands had to be cloned to obtain pure DNA sequence [3, 15, 24–26]. Furthermore, excised bands provide limited sequence data (generally <500 bp) [6, 20, 21, 33]. In some studies, no DNA sequence data at all has been obtained following DGGE analysis [5, 32, 34].

In this study, we have demonstrated the potential for combining cloning and DGGE into a single protocol that not only provides a profile of the whole community, but also facilitates screening of clones for sequencing. Either partial or near full-length 16S rDNA PCR fragments were cloned. Individual clones were screened using DGGE (which reflects the melting characteristics of the entire fragment) rather than restriction enzymes (which target only short recognition sequences of four or six bases). Clone pools consisting of 35–50 clones were created, and their DGGE profiles were compared to direct DGGE profiles, to provide a visual estimate of coverage of the library. A sufficient number of clones had been pooled when the direct DGGE and clone pool DGGE profiles converged. Yu and Morrison [33] discussed the relative merits of different hypervariable regions of 16S rDNA as targets for DGGE. Clone/DGGE would allow multiple regions to be targeted simultaneously. Once individual clones have been shown to comigrate with specific bands in the community profile, near full-length DNA sequence could be obtained from the archived clones rather than from the gel.

The direct DGGE profile of the humics reactor biofilm community (Fig. 2) was obtained from the original DNA extract using primers 1070F and 1392R + GC. However, the clone library was produced with primers 8F and 1492R. The 1070F/1392R + GC primer pair was then used to screen individual clones and the pool of 50 clones. Therefore, the genotypes amplified from the clone library were a subset of those previously amplified by the 8F/1492R pair. If the 8F/1492R pair had a narrower specificity than the 1070F/1392R pair, the first round of PCR would have limited the genotypes available for the clone pool DGGE profile. Nevertheless, the pool of 50 clones appeared to contain clones that matched most of the prominent bands in the direct DGGE profile (Fig. 2, major arrows). Because the community was somewhat simple and skewed toward the two dominant bands (each of which may not represent a single DNA sequence), clones matching the migration of these two bands were identified in the first sampling of 10 clones. Obviously, a brute force approach in which these 50 clones were sequenced without screening would have identified the genotypes in these two bands as well as several other rarer genotypes. However, much of that sequencing would have been redundant. The clone/DGGE strategy can minimize redundant sequencing, although it is always necessary to sequence a few clones having the same DGGE migration to confirm that the dominant genotype has been identified. We tentatively identified the two dominant genotypes with minimal sequencing.

All analysis of the freshwater pond community was performed using only the 1070F/1392R (or 1070F/1392R+GC) primer pair. This choice avoided potential biases from primer pairs with different specificities, but limited the length of DNA sequence contained in the clones. Both the clone pool and direct DGGE profiles indicated two distinct bands (Fig. 3). Clones matching the migration of these two bands were encountered in the first group of 12 clones screened (lanes 5 and 6). Therefore, random sequencing of up to 35 clones would have produced these same results, although with some redundant sequencing. Two different pools of 35 clones contained the same two dominant bands, but differed from each other in several other bands, some of which were not visible in the direct DGGE profile. The differences between the two pools may be explained by laws of probability. A somewhat rare genotype could easily end up in one pool but not the other.

The great inherent microbial diversity in soils (and rhizospheres) [29] is difficult to analyze by any method and would test the limits of the clone/DGGE protocol. Borneman and Triplett [4] argued that the differences between two (unreplicated) sets of 50 clones, one from a forest and the other from an adjacent pasture, were evidence of microbial community shifts during deforestation. Clone/DGGE provides a test for this conclusion by comparing clone pool and direct DGGE profiles. For example, whether 50 clones are enough to represent the diversity in a sample could be tested by comparison of the DGGE profiles of 50-clone vs 100-clone pools. This approach might serve as an alternative to rarefaction analysis, which plots the number of unique clone sequences detected vs the total number of clones sequenced [18, 35]. However, rarefaction analysis is after the fact and does not have clone/DGGE's potential for reducing redundant sequencing.

In analysis of the lawn soil microbial community, PCR products amplified using the 8F/1492R primer pair were used both for cloning and as template for the direct DGGE profile. Therefore, primer pair specificity was not responsible for differences between the clone/DGGE and direct DGGE profiles. Despite obvious differences between these profiles, some bands in the direct DGGE profile corresponded to bands from clones (Fig. 4, major arrows). Clone/DGGE would prove to be a superior method for obtaining DNA sequence information from soil microbial communities if clones comigrating with selected bands in the direct DGGE profile could be identified more easily and rapidly than excised gel bands could be processed for DNA sequencing. A strategy for identifying individual clones corresponding to any band would be, for example, to create an image containing DGGE profiles from 10 different 50-clone pools in separate lanes, interspersed with lanes of direct DGGE profiles to facilitate alignment. Once a pool had been shown to contain a desired clone, the clone pool could be divided into increasingly smaller subsets of clones. A final image would contain the direct DGGE profile along with individual clones.

Biases affecting molecular microbial community analysis are widely acknowledged, including DNA extraction biases [10], differences in rRNA gene copy number [7], and PCR amplification biases [23]. Obviously, these biases would affect both cloning and direct DGGE because these initial steps are common to both approaches. Polz and Cavanaugh [23] argued that PCR amplification biases could be minimized by avoiding primer degeneracies, by pooling amplification products from replicate PCR tubes, and by using both higher template concentrations and fewer PCR cycles (~10, compared to ~25 typically used). A case can be made that clone/DGGE may reduce PCR bias. Yu and Morrison [33] noted that using as little as 500 ng of template in a 10-cycle PCR reaction, a strategy for minimizing PCR bias, would yield enough product for cloning. It is unlikely, however, that the same amount of PCR product could be visualized using DGGE. Furthermore, with difficult samples, i.e., samples containing low biomass and/or contaminants and inhibitors of amplification, it would seem much easier to obtain enough PCR product for a cloning reaction than for DGGE analysis. Once a clone library has been created, contaminants and low biomass are no longer concerns. Clone libraries may be archived indefinitely.

In subsequent studies, we intend to investigate further the potential of clone/DGGE to facilitate the analysis of the complex microbial communities such as those found in soils. Part of the focus will be on high-throughput screening, including adoption of a 96-well format. In environments of great genetic diversity, 16S rDNA may not be the appropriate marker gene. It might be more informative to use DGGE to study the diversity of functional genes [3, 13]. Alternatively, the extracted DNA could be fractionated based on GC content prior to DGGE analysis [12]. Clone/DGGE could easily be adapted to either scenario and has potential for analysis of diverse microbial communities.

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