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Direct Extraction of DNA from Soils for Studies in Microbial Ecology

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Abstract

Molecular analyses for the study of soil microbial communities often depend on the extraction of DNA directly from soils. These extractions are by no means trivial, being complicated by humic substances that are inhibitory to PCR and restriction enzymes or being too highly colored for blot hybridization protocols. Many different published protocols exist, but none have been found to be suitable enough to be generally accepted as a standard. Most direct extraction protocols start with relatively harsh cell breakage steps such as bead-beating and freeze-thaw cycles, followed by the addition of detergents and high salt buffers and/or enzymic digestion with lysozyme and proteases. After typical organic extraction and alcohol precipitation, further purification is usually needed to remove inhibitory substances from the extract. The purification steps include size-exclusion chromatography, ion-exchange chromatography, silica gel spin columns, and cesium chloride gradients, among others. A direct DNA extraction protocol is described that has been shown to be effective in a wide variety of soil types. This protocol is experimentally compared to several published protocols.

Introduction

Environmental microbiologists are growing more dependent on enumeration and detection methods that use molecular biology techniques rather than traditional culture techniques. Enrichment media used to culture microbes in the laboratory are inherently selective and only a subpopulation of the microbes in an environmental sample will grow on any given medium. Even if several different media and growth conditions are used, many of the native microbes will not grow in the laboratory. Using traditional culture techniques to measure the biodiversity of an environmental sample will underestimate species richness and skew measurements of species evenness. The most often cited research addressing this issue suggests that less than 1% of microbes in the environment can be cultured in the laboratory using current methods (Torsvik *et al*., 1990). Although this value can be debated, it is generally accepted that culture-based methods miss much of the microbial diversity in environmental samples.

Microbial ecologists have turned to culture-independent methods for community analysis. Rather than isolating microbes, molecular biology techniques are used to isolate and compare the sequences of specific genes (Atlas *et al*., 1992; Madsen, 1998; Schneegurt and Kulpa, 1998). Taxonomic designations can be derived from phylogenetic analyses of rDNA gene sequences. Whole community DNA extracts are subjected to PCR amplification directed broadly at rDNA sequences or at the rDNA genes of specific clades of organisms. The resulting mixture of rDNA amplicons can be cloned into an appropriate vector and individual amplified rDNA genes methodically sequenced or segregated by RFLP analysis prior to sequencing. DGGE or SSCP analyses also can be used to separate rDNA amplicons that have similar lengths, but different melting points or secondary structures, and hence different primary sequences. Other types of fingerprinting analyses can be used as well. In any case, the success of the project depends on obtaining DNA extracts that are in sufficient quantity and of sufficient purity to be manipulated using these sensitive molecular biology protocols.

Soils present some of the most difficult challenges to the development of suitable extraction and purification procedures. The complex matrix of soil harbors a variety of substances that inhibit the activity of polymerases and restriction enzymes or interfere with hybridization and detection protocols (Steffan *et al*., 1988; Demeke and Adams, 1992; Tsai and Olson, 1992). Of particular concern are humic and fulvic acids found in great abundance in soils of high organic content that are subject to natural degradation. These fractions are such complex mixtures of related compounds that they demonstrate a broad spectrum of solubilities and charge characteristics. It is difficult, if not impossible, to find extraction and separation conditions that can remove all humic substances from soil DNA extracts. Notice that this review will present a wide range of methods for purifying DNA extracts, but none of these has been shown to be robust and general enough to be accepted by the scientific community as a standard protocol.

Extraction of DNA from soils requires no culturing or growth of the organisms. The procedures can be divided into two groups. In one case, soil samples are exposed to some mild physical and/or chemical disruptions meant to dislodge microbes adhered to soil surfaces (Torsvik, 1980; Holben *et al*., 1988; Steffan *et al*., 1988; Josephson *et al*., 1991; Jacobsen and Rasmussen, 1992; Holben 1994; Courtois *et al*., 2001). The free microbes are then separated from the soil matrix before DNA extraction begins. The

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second and more popular method is the direct extraction of DNA from soil. Harsh physical and/or chemical disruptions are used to break open cells while still in the soil matrix. The DNA is then extracted and purified from this messy mixture. Each of these methods have both positive and negative aspects in terms of their efficiency and ability to generate DNA extracts that truly represent the natural microbial community.

Sample Handling Issues

The way that samples are taken, transported, and stored prior to extraction is important for those interested in studying the microbial ecology of native communities. By its very nature, sampling soils physically disrupts soil structure in a way that can alter the microbial community. Therefore, it is important to extract or freeze the samples as quickly as possible. The best method is to freeze samples on dry ice taken to the field site and then to store the samples in an ultralow freezer in the laboratory. Unfortunately, there are many published works where soil samples were stored on the benchtop for long periods or stored at 4 °C for hours or days before extraction. This kind of storage fundamentally changes the microbial community and results obtained using molecular phylogeny methods will not accurately describe the original native community. For example, Pseudomonads tend to proliferate and come to dominate samples left in a refrigerator, even over short periods of hours or days. The resulting community does not resemble the original community.

It is clear that microbial communities in soils vary greatly over small spatial and temporal scales. Composite sampling where several (five or more) small samples are collected in a limited area (1 m^2) is a good method for damping the variations. Adequate mixing is necessary and extraction should be performed on gram quantities of soil to reduce nugget effects. Often soils need to be sieved (2 mm) before extraction to remove large debris and rocks. This can be done when frozen samples are thawed prior to extraction. It is important that as little manipulation as possible is done in the field before freezing the samples to limit changes in the community due to physical disruption.

One of the more challenging aspects of sampling soils in the field is cleanliness. It is difficult, if not impossible, to take samples using aseptic techniques in the field. Sampling materials like corers, spades, and spatulas can be sterilized by autoclaving and/or rigorous UV irradiation to limit contamination of the samples. However, windblown materials can be a considerable problem in the field. One must also be sensitive to the distinctions between autochthonous and allochthonous microbes. For example, if a site is subject to flooding, defining which organisms are native to the flood plain and which were recently deposited from the stream becomes difficult.

Cell Breakage

Direct DNA extraction methods all include steps to break microbial cells within the soil matrix. Microbes are not separated from soil or grown in culture before breaking the cells to release their DNA. Many protocols include several breakage steps that each use a different physical or chemical mechanism. There are significant issues associated with breakage methods that influence the quality of the data obtained from the DNA extracts. It is generally accepted that the great advantage of culture-independent methods of examining microbial communities is that these are unbiased by the ability or inability of particular species to grow in culture and hence give a more accurate picture of the native community. However, particular breakage regimes could bias the content of the DNA extract toward specific groups of bacteria. Gram-negative cells tend to break more easily than Gram-positive cells. Thus, more mild breakage regimes may not sufficiently break Gram-positive cells or their spores and can potentially enrich the extracts with DNA from Gram-negative organisms. However, since Gram-negative cells break open more easily, harsher breakage regimes may significantly shear the released DNA. Thus, harsher methods may enrich for DNA from Gram-positive organisms. In many cases, fairly small DNA fragments are targeted for amplification, so that shearing is not as important. Most laboratories use relatively harsh breakage regimes.

The most commonly used protocols are based on that of Tsai and Olsen (1991). Here freeze-thaw cycles are used to break the cells in soil before DNA extraction (Picard *et al.*, 1992; Erb and Wagner-Döbler, 1993; Herrick *et al.*, 1993; Moré *et al.*, 1994; Cullen and Hirsch, 1998; Edgcomb *et al.*, 1999). Soil slurries are alternately rapidly frozen in a dry ice-acetone, dry ice-ethanol, or liquid nitrogen bath and then rapidly thawed in a 60 to 100˚C water bath. Bead-beating is another popular cell breakage method (Ogram *et al.*, 1987; Smalla *et al.*, 1993; Tebbe and Vahjen, 1993; Holben, 1994; Moré *et al.*, 1994; Cullen and Hirsch, 1998; Courtois *et al.*, 2001). Soil slurries are supplemented with small zirconia/glass beads and then violently shaken at more than 500 rpm to physically disrupt cells. Modified vortex mixers or specially design shakers can be used. These are generally smaller and less sophisticated versions of the long-lived carbon dioxide-cooled Braun homogenizers. The size of the beads used, the period of milling, and the breakage buffer composition vary between protocols. In general, an equal volume of beads (0.1 to 0.2 mm) and soil are added to a breakage medium that may contain detergents and high salt concentrations and the mixture is milled for 3 to 15 minutes, often in the cold. Direct grinding of soils in liquid nitrogen using a mortar and pestle (Volossiouk *et al.*, 1995; Ranjard *et al.*, 1998; Edgcomb *et al.*, 1999) and ultrasonication (Picard *et al.*, 1992) also have been reported.

Chemical breakage methods are used in combination with physical methods in many protocols. The most popular detergent treatment includes SDS at 1% and salt concentrations of 1 M or more, often coupled with heating and shaking (Steffan *et al.*, 1988; Bruce *et al.*, 1992; Herrick *et al.*, 1993; Smalla *et al.*, 1993; Holben, 1994; Lovell and Piceno, 1994; Moré *et al.*, 1994; Volossiouk *et al.*, 1995; Porteous *et al.*, 1997; Cullen and Hirsch, 1998; Edgcomb *et al.*, 1999). A hot-SDS lysis method was first presented by Selenska and Klingmüller (1991). A persistent problem with soil extractions is that DNA tends to adsorb to soil

particles giving lower yields (Greaves and Wilson, 1969; Lorenz and Wackernagel, 1987; Ogram *et al.*, 1988). Adding detergents and salts can help to alleviate this problem, although SDS can inhibit PCR if not removed in subsequent steps (Weyant *et al.*, 1990). Some protocols, particularly commercially available kits, tend to use strong chaotropic agents like guanidinium salts (Tsai and Olson, 1990; Porteous *et al.*, 1997). Degradatory enzyme digestion steps are often included in breakage regimes. Incubation with lysozyme (Hilger and Myrold, 1991; Rochelle and Olson, 1991; Tsai and Olson, 1991; Erb and Wagner-Döbler, 1993; Herrick *et al.*, 1993) followed by incubation with a protease (Porteous and Armstrong, 1991; Tebbe and Vahjen, 1993; Ranjard *et al.*, 1998; Courtois *et al.*, 2001) often precedes the addition of detergent and salt. Although these steps may help to liberate DNA from cells, a cautionary note must be added suggesting that DNA degradation may occur during the long (an hour or more) and warm (37°C) enzymic digestions.

Our laboratory has had success with a breakage regime that includes many of these elements. Soil slurries are first subjected to bead beating (with 0.1 mm beads) in a simple phosphate-buffered solution containing 1% SDS. Proteinase K is added to 100 µg ml^{-1} and incubated for 1 h at 37˚C. This is followed by 4 cycles of freeze-thaw using a dry ice-ethanol bath and an 80˚C water bath (3 min in each per cycle). Direct counting of cells with acridine orange staining and direct plating experiments demonstrated that in excess of 90% of the observable cells and viable cells were broken using this protocol.

Nucleic Acid Extraction

The extraction and collection of nucleic acids from the soil homogenates generally follow standard protocols that include organic extraction and alcohol precipitation (Sambrook *et al.*, 1989). The soil homogenates can be centrifuged to remove soil debris, but often the extractions proceed directly with the homogenate. Many protocols facilitate breakage and the subsequent deproteination step by adding CTAB and increasing the salt concentration (Zhou *et al.*, 1996; Porteous *et al.*, 1997; Ranjard *et al.* 1998; Edgcomb *et al.*, 1999). This may also help remove humic materials. Our protocol brings the sodium chloride concentration to 4.5% and the CTAB concentration to 1.2% followed by a 20-min incubation at 65˚C. Organic extraction with a variety of phenol and chloroform combinations is included in the extraction protocols to deproteinate the homogenate and remove a good deal of the humic and pigmented components. In some cases, phenol extraction(s) is followed by chloroform extraction, while others extract with a mixture of phenol and chloroform. We extract homogenate supernatants with Tris-buffered phenol (pH 8.0) and then with water-saturated chloroform:isoamyl alcohol solution (24:1).

Nucleic acids are precipitated from the aqueous phase of the organic extractions by the addition of alcohol. The soil extraction protocols use either isopropanol (one volume) or ethanol (two volumes). In most cases, the mixture is cooled, often overnight, before centrifugation to collect nucleic acids. The pellets, which are usually large and darkly colored from the humic constituents, are dried in vacuo and resuspended in a small amount of buffer. Resuspension can be slow and usually requires heating (65˚C for 1 h) or prolonged incubation (overnight at 4˚C). These crude DNA preparations can often be used for molecular analyses, but in most cases are too contaminated with enzyme inhibitors for amplification by PCR or too highly colored for blot hybridizations. Extractions from sediments and other soils with low organic contents are less troublesome. In some cases, extensive dilution of the extract will allow for direct PCR amplification from these crude DNA extracts.

DNA Purification

It is at this point that the published protocols tend to greatly diverge. Crude DNA extracts from soils are usually too impure to allow for molecular analyses and need to be further purified. There is no agreement as to the most effective method of purification. Many of the protocols appear to be idiosyncratic and only effective on the soil type for which they were developed. Other protocols claim to be more generally applicable, but results in individual laboratories vary considerably. We have tried several common protocols with a variety of soils and will report about our experiences here.

The use of some type of silica gel or silica membrane separation is perhaps the most popular purification step (Porteous and Armstrong, 1991; Smalla *et al.*, 1993; Moré *et al.*, 1994; Zhou *et al.*, 1996; Ranjard *et al.*, 1998). Many commercially available clean-up kits such as Promega’s Wizard DNA clean-up kit and the Bio101 (Vista, CA) FastDNA Spin kit (analogous to Gene-Clean) use silica gel spin columns to remove humic materials from DNA extracts. Silica gel (glass milk) can be purchased separately and used without purchasing entire kits. The silica gel binds to humic materials and DNA initially, and some humic acids can be sequentially eluted from the matrix. This same technology is found in the Elutip d syringe-tip filters used by Picard *et al.* (1992). Our protocol includes two silica gel mini-columns as the final polishing steps. After organic extractions and alcohol precipitation, our DNA is purified by passage through a DEAE-cellulose column, reprecipitated, and then passed through a Wizard DNA clean-up column and a Bio101 FastDNA spin column. As discussed below, we did not find the silica gel protocols alone to be effective in removing sufficient amounts of humic materials from DNA extracts of several soil types.

Although not as commonly used, we found ion-exchange chromatography to be an excellent way to remove humic materials from soil DNA extracts. DEAE-cellulose columns are commonly used for purifying tRNAs and have been used for DNA extracts (Holley *et al.*, 1961; Lovell and Piceno, 1994). Hydroxyapatite column chromatography has been used effectively as well (Markov and Ivanov, 1974; Ogram *et al.*, 1987; Steffan *et al.*, 1988). These column steps are relatively laborious, but the great amount of humic materials removed makes them worthwhile. Syringe-tip ion-exchange columns are available commercially and have been applied to DNA purification from soils (Tebbe and Vahjen, 1993). In our laboratory,

Table 1. Soil Descriptions

Soil	Type	% Moisture	% Organic Matter	Metal Content (µg per g dry wt. soil)				
				Ca	Fe	Mg	K	Na
Bendix Woods	Silty clay loam	28.8	10.4	7,446	17,595	2,639	17,504	2,576
St. Mary's Lake	Loamy sand	25.1	13.6	70,667	62,038	10,440	10,958	2,088
Yellowstone Forest	Clay loam	43.3	46.8	6,341	11,240	2,269	15,800	5,959
Rainforest	Silt loam	43.5	22.0	7,456	87,899	5,774	7,495	5,863
Spinn Prairie	Silty clay	13.5	6.0	6,117	10,978	2,362	14,589	4,340
Cornfield	Sandy loam	8.4	4.8	8,224	26,517	5,101	18,688	4,420
Desert Sand	Loamy sand	3.4	2.9	23,622	49,219	24,567	34,015	14,860
Marine Sediment	Sand	20.6	0.9	7,656	17,174	4,072	10,519	3,642
Peat	Peat	90.2	75.0	1,388	447	159	846	1,905
Yellowstone Clay	Clay	29.1	7.0	2,068	14,860	1,710	15,980	1,807

crude DNA extracts are applied in a low-salt buffer (0.1 M NaCl) to small (2 ml) DEAE-cellulose beds held in disposable fritted columns. The DNA is preferentially eluted when the salt concentration of the eluant is raised to 0.5 M. Some humic materials are eluted with the DNA, but the vast majority remains bound to the matrix. In most cases, the DNA extracts obtained after DEAE-cellulose chromatography are not sufficiently pure for PCR or restriction enzyme digestion. The eluted DNA is precipitated with alcohol to reduce its volume and remove salts in preparation for the final silica gel spin column procedures.

Another popular purification method is the use of agarose gel electrophoresis to separate DNA from humic materials (Hilger and Myrold, 1991; Rochelle and Olson, 1991; Herrick *et al.*, 1993; Zhou *et al.*, 1996). Polyacrylamide and dextran gel filtration columns also use size as a basis for separation (Tsai and Olson, 1992; Erb and Wagner-Döbler, 1993; Jackson *et al.*, 1997; Cullen and Hirsch, 1998; Ranjard *et al.*, 1998; Edgcomb *et al.*, 1999). Jackson *et al.* (1997) compared the effectiveness of Sepharose 4B, Sephadex G-200, and Sephadex G-50 with a diverse set of soils and found Sepharose 4B to be superior. Membrane-based microconcentrators can be used for size separations (Porteous *et al.*, 1997). Molecular sizing will remove much of the humic materials, but since this is such a diverse mixture of materials, a significant amount of humic materials comigrate with the DNA band. Additional purification is often needed. In some cases, a strip of gel containing polyvinylpolypyrrolidone (PVPP) is incorporated a short distance from the wells of an agarose gel. The DNA is unimpeded upon passage through the PVPP strip, while humic materials can be trapped. In other cases, the PVPP is included throughout the gel (Herrick *et al.*, 1993). Some researchers have used PVPP powder in batch or in spin columns to bind humic materials and purify DNA extracts (Steffan *et al.*, 1988; Hilger and Myrold, 1991; Porteous and Armstrong, 1991; Picard *et al.*, 1992; Zhou *et al.*, 1996; Cullen and Hirsch, 1998). Powdered milk also has been used to adsorb humic materials during extractions (Volossiouk *et al.*, 1995).

A very traditional method, cesium chloride gradients, appears to be very effective (Ogram *et al.*, 1987; Steffan

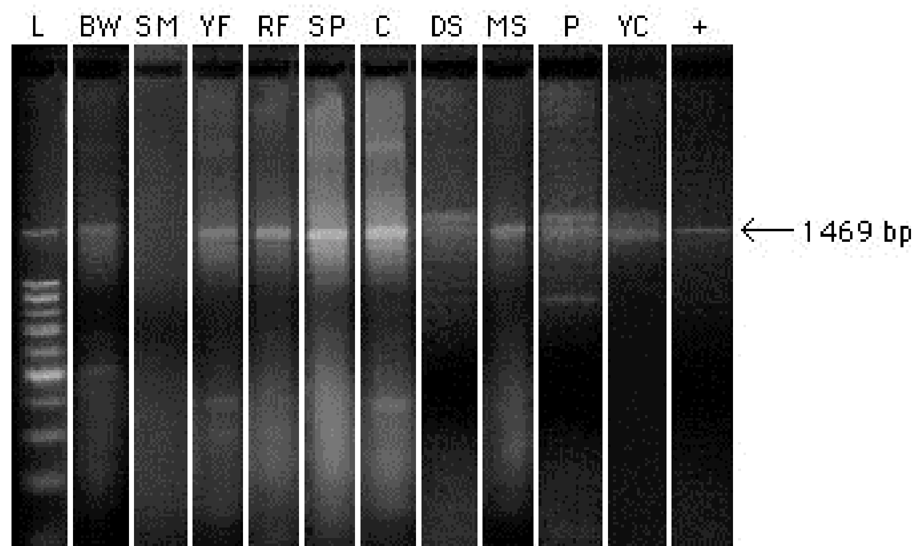

Figure 1. Gel electrophoretic separation of DNA fragments obtained by PCR amplification of 16S rDNA genes in soil extracts produced by the current protocol. L = 100-bp DNA ladder, RF = rainforest soil, YF = Yellowstone Forest, P = peat, YC = Yellowstone clay, C = cornfield soil, BW = Bendix Woods soil, MS = marine sediment, DS = desert soil, SP = Spinn Prairie soil, SM = St. Mary's Lake soil, + = pure *P. putida* culture.

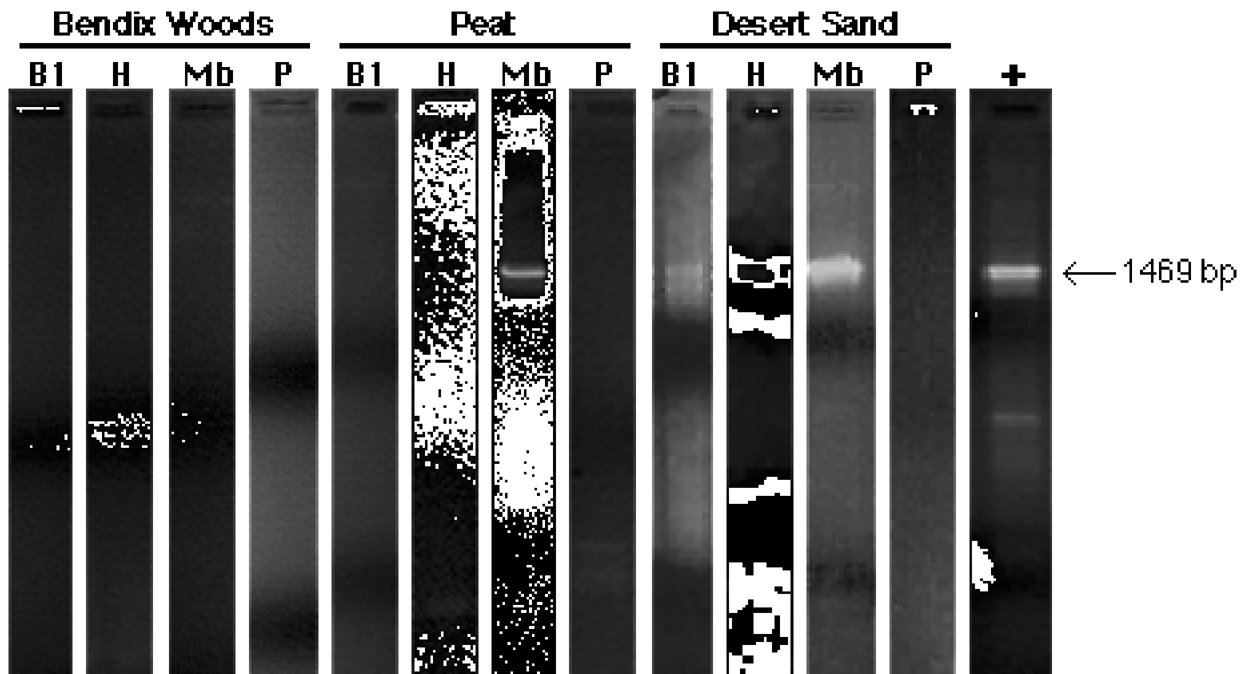

Figure 2. Gel electrophoretic separation of DNA fragments obtained by PCR amplification of 16S rDNA genes in soil extracts produced by published and commercial protocols. B1 = Bio101 FastDNA spin kit for Soil, H = protocol from Herrick et al. (1993), Mb = MoBio Inc. Ultraclean Soil DNA kit, P = protocol from Picard *et al.* (1992).

et al., 1988; Holben *et al.*, 1988; Porteous *et al.*, 1991; Selenska and Klingmüller, 1991; Bruce *et al.*, 1992; Jacobsen and Rasmussen, 1992; Smalla *et al.*, 1993; Holben, 1994; Lovell and Piceno, 1994; Courtois *et al.*, 2001). These are rather labor intensive protocols that work best with relatively large quantities of DNA. There have been suggestions that differential ethanol precipitations can be used to remove humic materials. Precipitations with 0.1 to 0.5 volumes of ethanol remove some humics. We have not found this protocol to be very effective in the variety of soil types we tested.

Comparison of Protocols

The DNA extraction protocol used in our laboratory gave extracts pure enough for PCR amplification from a wide range of soils. A PCR primer set directed against Eubacterial 16S rDNA sequences that generates a 1469-bp amplicon (pA, pH from Edwards *et al*., 1989) was used to test the effective purity of the DNA extracts. PCR was performed in 100-µl reactions using 1 µl of the DNA extracts, and the products were tested by hybridization with an internal probe. The ten soils tested widely differed in type, organic matter content, and metal concentrations, being derived from a disparate set of environments (Table 1). The organic content of the soils ranged from 75% organic matter in peat to 0.9% organic matter in marine sediment. Although peat had the highest organic content, it was relatively undegraded material, while the forest soils apparently had the greatest levels of humic materials. Nine of the ten soil DNA extracts were of sufficient purity and abundance to PCR amplify bacterial 16S rDNA sequences (Figure 1).

Three of the soils were chosen for further study, a forest soil, a desert sand, and peat. Four previously reported DNA extraction protocols were applied to these soil, that of Herrick *et al.* (1993), Picard *et al.* (1992), the MoBio Ultraclean Soil DNA Kit, and the Bio101 FastDNA SPIN Kit for soil. The commercial kits essentially rely on silica gel spin columns for purification of the DNA. The Picard protocol includes PVPP in the breakage buffer and uses three successive passages through Elutip d columns (Schleicher and Schuell Dassel, Germany). The Herrick protocol uses PVPP in an agarose gel for electrophoretic purification of the DNA. Three of the extraction protocols tested generated PCR amplicons from the desert sand (Figure 2). The Herrick protocol gave no visible products from desert sand. No visible PCR products were generated from the forest soil. The MoBio procedure yielded a suitable extract from peat. The Picard procedure also gave a faint positive reaction with peat extracts. In our hands, these protocols worked well for soils that did not have high humic contents, but were unable to provide DNA extracts that were suitable for PCR from soils rich in humic materials.

To distinguish between extracts that were poor due to inhibitory substances from those that simply did not have sufficient yields to be effective, an inhibition assay was developed where a known amount of a specific functional gene, the *xylE* gene from *Pseudomonas putida* mt-2, was amplified in the presence of soil DNA extracts. When the Bio101 or MoBio protocols were used to extract DNA, the extracts obtained from Bendix Woods soil and from peat were inhibitory to PCR when 1 µL was added to the 100-µL reaction volume, while the extracts from sand were not inhibitory when 5 µL were added. The Picard *et al.*

Table 2. Yield and metal content of DNA extracts

Soil	Yield	Fe Content	Ca Content
	(μg per g dry wt. soil)		
Bendix Woods	33.8	0.0837	0.3725
St. Mary's Lake	35.3	0.0326	0.2069
Yellowstone Forest	25.8	0.0268	0.3100
Rainforest	28.5	0.0372	0.3388
Spinn Prairie	13.8	0.0251	0.1526
Cornfield	7.9	0.0465	0.1946
Desert Sand	nd	0.0468	1.1595
Marine Sediment	3.7	0.0453	0.2163
Peat	nd	0.0526	0.3947
Yellowstone Clay	1.7	0.0312	0.3209

procedure yielded an extract from Bendix Woods soil that was inhibitory to PCR at the 1-μL level, but the peat and sand extracts were not. None of the extracts obtained using the Herrick procedure were inhibitory to PCR. Most of the extracts obtained by the new protocol were not inhibitory to PCR even when 5 μL were added. One μL of DNA extract obtained using the current protocol on St. Mary's Lake soil was enough to inhibit PCR and 3 μL of the Yellowstone forest soil also was inhibitory. The protocol developed by Herrick *et al.* did not result in an extract that was inhibitory to PCR , but still no PCR products were obtained with this protocol. A likely explanation for this is that this procedure resulted in a low yield of DNA.

The yield of DNA using the current protocol varied greatly with soil type, ranging from 25 to 35 μg DNA per gram dry weight soil for forest soils to 1.7 μg DNA per gram dry weight soil for the Yellowstone clay (Table 2). The yield of DNA obtained by the current protocol was generally similar to reported values (Tebbe and Vahjen 1993, Zhou *et al.* 1996, Frostegård *et al.* 1999 [non-purified yields], Miller *et al.* 1999). Two soils gave yields of DNA that were lower than the rest of the soils tested. One was the Marine Sediment which has very low organic content and likely low biomass abundance. The other soil giving a low yield was the Yellowstone Clay. Frostegård *et al.* (1999) found that DNA adsorbs strongly to soil colloids in clay-rich soil

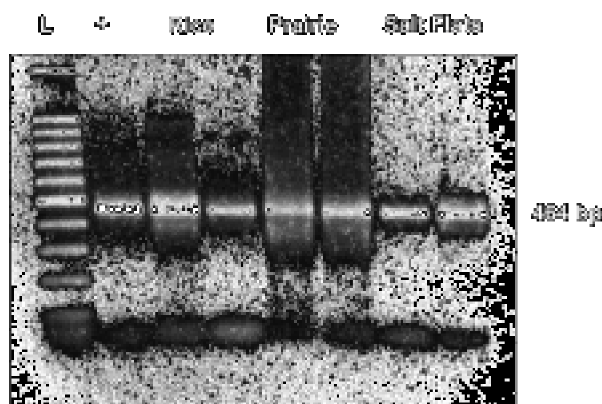

Figure 3. Gel electrophoretic separation of DNA fragments obtained by PCR amplification of *nifH* genes in DNA extracts produced by the current protocol from two ricefield soils, two prairie soils, and two salt flats hypersaline soils. L = 100-bp DNA ladder, + = pure *Anabaena* culture.

and were unable to recover any DNA from clay-rich soil even after seeding it with up to 50 μg of DNA.

The sensitivity of our soil extraction protocol was determined by seeding soil samples prior to extraction with known amounts of bacteria that contain a kanamycin-resistance gene unlikely to be abundant in nature. We were able to detect the presence of bacteria when added to Bendix Woods soil at 10^2 cells·gram^{-1}. Tsai and Olson (1992) were able to detect 2 x 10^5 cells·gram^{-1} from soil rich in humic acids. Degrange and Bardin (1995) were able to detect 10^2 *Nitrobacter sp.* cells from 0.6 grams of soil having a high organic content. Picard *et al.* (1992) reported a detection limit of 10^4 cells·gram^{-1} from moderately organic soil. Berthelet *et al.* (1996) reported a sensitivity of between 10^1 and 10^2 cells·gram^{-1} of forest soil, however, they used nested PCR to achieve this sensitivity. Cullen *et al.* (1998) were able to detect 300 genetically modified Rhizobia cells·gram^{-1} soil of unknown organic content. Smalla *et al.* (1993) were able to detect 3 x 10^3 cells·gram^{-1} of soil using an organism that had 3 copies of the target gene per cell. Although the seeding assay used in the current study gave a sensitivity that was quite high, the sensitivity of the current protocol for detecting native bacteria in soil may be somewhat lower since indigenous soil bacteria are more intimately associated with soil particles, and therefore harder to break than cells grown in pure culture and then added to soil (Cullen *et al.* 1998).

We also have been successful in amplifying functional genes from unseeded soils (Figure 3). These genes are naturally in lower abundance than rDNA genes and only found in a small subset of the bacteria in any particular soil sample. Using primers directed at the *nifH* gene encoding the small subunit of nitrogenase (Zehr and McReynolds, 1989; Ueda *et al.*, 1995), we generated the expected 464-bp fragment with soil extracts from rice paddies at Southeast Missouri State University, tallgrass prairie from the Ninnescah field station at Wichita State University, and a hypersaline sandy soil from the Great Salt Plains of Oklahoma. Clone libraries for RFLP analyses and sequencing can be generated from these PCR amplicons and the diversity of functional guilds estimated.

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