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Amplification of DNA from Native Populations of Soil Bacteria by Using the Polymerase Chain Reaction

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Specific DNA sequences from native bacterial populations present in soil, sediment, and sand samples were amplified by using the polymerase chain reaction with primers for either "universal" eubacterial 16S rRNA genes or mercury resistance (*mer*) genes. With standard amplification conditions, 1.5-kb rDNA fragments from all 12 samples examined and from as little as 5 μg of soil were reproducibly amplified. A 1-kb *mer* fragment from one soil sample was also amplified. The identity of these amplified fragments was confirmed by DNA-DNA hybridization.

Increasing awareness of the contribution of bacteria to environmental processes, coupled with the need to monitor these environments for pathogens (14) and released genetically modified organisms (15), requires improved detection methods. Traditional methods that rely on bacterial cultivation identify only a small fraction (0.01 to 10%) of the total microbial biomass (9, 11, 22). Techniques that involve DNA extraction (e.g., references 21 and 23), followed by either direct DNA probing (10) or amplification by the polymerase chain reaction (PCR), are being developed as more sensitive ways to assess the microbial content of natural environments. In aquatic environments, DNA probing has been used successfully to detect bacterial mercury resistance (*mer*) genes (1, 2) and native bacterial genes have been amplified and sequenced without recourse to prior cultivation (5, 14, 18). However, this has not been possible for native soil bacteria without prior cultivation because of inhibition of PCR amplification by substances, such as humic acids, coextracted with DNA (3, 24). Amplification of DNA from soils has been reported, but only when amended by addition of the target to be amplified, e.g., transposon Tn*5* (16), chromosomal sequences from *Pseudomonas cepacia* (20), and 16S rRNA gene fragments (24). We report here a reproducible method suitable for PCR amplification of native bacterial rRNA and *mer* genes in DNA extracted from a variety of natural soils.

(These results were communicated by poster at the meeting of the Society for General Microbiology, Cardiff, Wales, March 1992.)

Soil, sediment, and sand samples were collected in the United Kingdom from Merseyside, Avon, and Wales and stored at 5°C. All reagents and containers were autoclaved and/or rigorously UV irradiated. DNA was extracted by using a modification of the method of Selenska and Klingmüller (19). Two grams (wet weight) of a sample, suspended in 5 ml of extraction buffer (1% sodium dodecyl sulfate [SDS] in 0.12 M Na_2HPO_4, pH 8.0), was incubated at 70°C for 1 h with occasional shaking and centrifuged at 2,800 × *g* for 10 min, and the supernatant was kept at 4°C. After two further extractions with 5 ml of fresh extraction buffer, the three supernatant fractions were pooled and centrifuged at 8,000 × *g* for 30 min, and the final supernatant was added to polyethylene glycol (final concentration, 15% in 10 mM Tris–1 mM EDTA buffer, pH 8.0) and 5 M NaCl (to 10% of the centrifuged fluid volume). After overnight precipitation at 4°C and centrifugation (5,000 × *g* for 10 min), the pellet was resuspended in 8 ml of 10 mM Tris–1 mM EDTA, giving a brown solution to which 8 g of CsCl and 100 μl of ethidium bromide at 10 mg ml^{-1} were added. After centrifugation for 18 h at 50,000 rpm and 18°C in a Beckmann Ti75 rotor, the single DNA band was withdrawn and the ethidium bromide was removed with CsCl-saturated isopropanol. Samples were dialyzed overnight in 10 mM Tris–1 mM EDTA. DNA was extracted with an equal volume of phenol-chloroform and precipitated overnight with 0.1 volume of 3 M sodium acetate (pH 4.8) and 2.5 volumes of 100% ethanol. Following centrifugation, DNA was washed repeatedly in 70% ethanol at room temperature to remove most of the remaining brownness and suspended in 400 μl of sterile distilled water. Purified chromosomal DNA from *Escherichia coli* AB1157, extracted with guanidium thiocyanate (17), and plasmid pACYC184::Tn*501*, extracted by the alkaline lysis method (4), were used as control sources of rRNA and *mer* genes, respectively.

"Universal" eubacterial primers pA (5′-AGA GTT TGA TCC TGG CTC AG-3′) and pH′ (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used to amplify 1.5 kb of rDNA (7). One kilobase of the *mer* operon containing *merRTΔP* was amplified by using primers jA (5′-GGG AGA TCT AAA GCA CGC TAA GGC [GA]TA-3′) and jB (5′-GGG GAA TTC TTG AC[TA] GTG ATC GGG CA-3′), which are based on the consensus sequence of conserved regions of the Tn*501*, Tn*21*, and pMJ100 *mer* determinants (12; unpublished data). Pairs of primers (20 pmol of each) were added to 50 μmol of a deoxynucleoside triphosphate mixture (dATP, dCTP, dGTP, and dTTP), together with target DNA, 2.5 U of *Taq* DNA polymerase (Boehringer Mannheim), and 10 μl of the supplier's 10× *Taq* DNA polymerase buffer, and reaction volumes were made up to 100 μl with sterile distilled water. The DNA weights used in PCRs are given in the figure legends.

Temperature cycling (with a Techne PHC 3 or a Genetic Research Instruments PTC-100 V2.0) for the rRNA gene fragment was 26 cycles of 94°C for 40 s, 55°C for 1 min, and

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72°C for 2 min. This was followed by one cycle of 72°C for 10 min. For the *mer* genes, the method differed only in the use of 62°C for the annealing step and 30 cycles of amplification. Reaction samples (10 μl) were electrophoresed through 0.7% agarose gels by using Tris-borate-EDTA buffer containing ethidium bromide.

The 18-bp DNA sequence pD′ (GTA TTA CCG CGG CTG CTG), previously used as a "universal" primer for rRNA genes (7), was used in this study to probe for rRNA genes and was prepared by end labelling of 1 μl of pD′ at 10 pmol μl^{-1} with 1 μl (10 μCi) of [γ-^{32}P]dATP–1 μl of 10× kinase buffer–10 U of T4 polynucleotide kinase–6 μl of distilled water at 37°C for 60 min. Unincorporated label was removed by using a NICK column (Pharmacia). The *mer* probe, a 2,217-bp *Hin*dIII-*Eco*RI fragment from Tn*501* containing the *merT* and *merP* genes, together with flanking regions of *merR* and *merA* (*mer* probe), was labelled with [α-^{32}P]dCTP by random priming and purified by using a Sephadex G50 column (8, 13).

Prior to vacuum blotting onto a GeneScreen membrane (DuPont), agarose gels were treated for 30 min with 0.2 M NaOH–0.6 M NaCl at room temperature and washed three times in blotting buffer (0.025 M Na_2HPO_4, NaH_2PO_4, pH 6.5) for 1 h at room temperature. Transferred DNA was bound by using 1,200 mJ of UV light (Stratalinker; Stratagene).

For hybridizations with pD′, the membrane was prehybridized at 40°C in 2% (wt/vol) blocking reagent (Boehringer Mannheim; prepared as a 10% solution in 100 mM maleic acid–150 mM NaCl, pH 7.5) with 5× SSPE–0.02% SDS–0.1% *N*-laurylsarcosine–20% (vol/vol) deionized formamide (5× SSPE contains 0.9 M NaCl, 0.05 M NaH_2PO_4, and 0.005 M EDTA, pH 7.7). After hybridization overnight at 40°C in 5× SSPE–0.02% SDS–0.1% *N*-laurylsarcosine–20% (vol/vol) deionized formamide, the membrane was washed five times, for 5 min each time, with prehybridization solution without blocking reagent at room temperature prior to exposure to Fuji RX 100 film with a Hi-Speed-X intensifying screen at −70°C. Hybridization of *mer* genes was done as recommended in the GeneScreen instruction manual (DuPont) but at 65°C instead of 60°C to provide greater stringency, i.e., detection of homology greater than 70%. Membranes were exposed to Fuji RX film at −70°C.

Following CsCl-ethidium bromide gradient centrifugation, the DNA extracted from all environmental samples produced a single fluorescent band containing between 2 and 21 μg of purified DNA per g of soil and having a size in excess of 12 kb (Fig. 1a, lane 1). No band was observed, nor was any DNA recovered, from the analogous position with autoclaved soil samples. In confirmation of earlier findings (19), all DNA samples were sensitive to DNase I and were cleaved by *Eco*RI, producing overlapping fragments (data not shown). However, brown pigment persisted, indicating that some impurities remained.

Figure 1a (lanes 7 to 12) shows amplification of 1.5-kb DNA fragments, with the pA and pH′ primers, from DNA extracted from Fiddler's Ferry soil (north side of the River Mersey). These are equivalent in size to that amplified from *E. coli* DNA (lane 5) with the same primers. Maximal PCR amplification occurred at 0.02 to 0.05 μg of DNA per 100-μl reaction (Fig. 1a, lanes 9 and 10). This is probably the optimal balance between the concentrations of target DNA and residual PCR-inhibitory substances. For this soil, the lowest concentration at which amplification was detected was 10^{-5} μg of DNA (data not shown). No band was observed following PCR of sterile distilled water (Fig. 1a, lane 6). As further controls to test for possible contamination of glassware and solutions by rDNA sequences, distilled water and autoclaved soil samples were separately processed by the DNA extraction procedure and gave no detectable amplification signal with the primers designed to amplify rRNA genes.

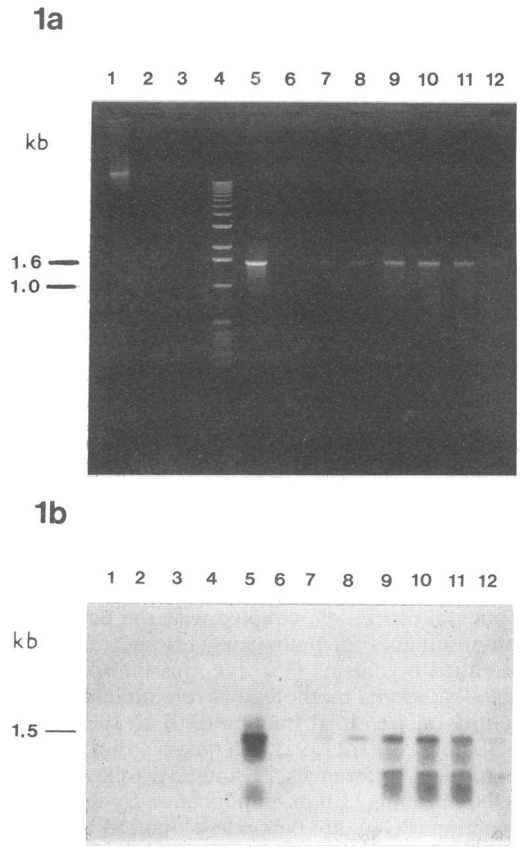

FIG. 1. (A) Agarose gel electrophoresis of the PCR products from Fiddler's Ferry soil DNA amplified with rRNA gene primers pA and pH′. Lanes: 1 to 3, 0.25, 0.02, and 0.0001 μg of soil DNA, respectively, without PCR; 4, kilobase ladder; 5, 0.48 μg of *E. coli* AB1157 DNA after PCR; 6, sterile distilled water after PCR; 7 to 12, 0.0001, 0.001, 0.02, 0.05, 0.10, and 0.25 μg of soil DNA, respectively, after PCR. (B) Southern blot analysis of the agarose gel shown in panel a hybridized to rRNA gene probe pD′. Lanes are numbered as in panel a.

All of the 1.5-kb PCR products amplified by primers pA and pH′ from either *E. coli* (Fig. 1b, lane 5) or soil DNA (Fig. 1b, lanes 7 to 12) hybridized to the pD′ probe. However, no hybridization was observed with nonamplified DNA loaded at three concentrations used for PCR amplification (Fig. 1b, lanes 1 to 3), illustrating the greater sensitivity of the PCR method and the specificity of pD′. This sequence homology confirms the amplification of native eubacterial rRNA genes present in soil DNA. Eubacterial rRNA genes were PCR amplified from DNA extracted from all of the environmental samples examined (eight different soils, three sediments, and one sand).

PCR products have been detected from only two of the native DNA extracts with the *mer* primers. Both samples were taken from the River Mersey estuary, which is known to have high mercury levels (6). Figure 2a shows a 1-kb

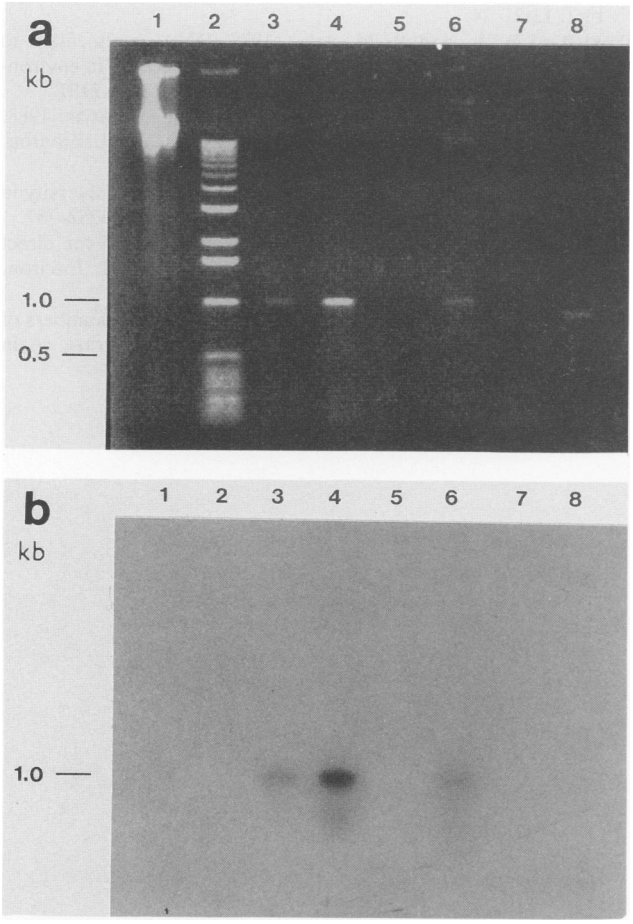

FIG. 2. (A) Agarose gel electrophoresis of the PCR products of soil and sediment DNA amplified with *mer* primers jA and jB. Lanes: 1, 0.82 μg of Fiddler's Ferry soil DNA without PCR; 2, kilobase ladder; 3 and 4, 0.03 and 0.07 μg, respectively, of Fiddler's Ferry soil DNA (separate extracts) after PCR; 5, sterile distilled water after PCR; 6, 0.14 μg of Tn*501* DNA after PCR; 7, 0.04 μg of Avon soil DNA after PCR; 8, 0.002 μg of DNA from sediment from the south side of the River Mersey after PCR. DNA concentrations for native DNA are those that gave optimum amplification. (B) Southern blot analysis of the agarose gel shown in panel a hybridized to the *mer* probe. Lanes are numbered as in panel a.

fragment amplified from two separate DNA extractions from Fiddler's Ferry soil (lanes 3 and 4); this fragment was identical in size to that amplified from Tn*501* DNA (lane 6) and an approximately 800-bp product from sediment from the south side of the Mersey (lane 8). No amplification was observed from sterile distilled water (Fig. 2a, lane 5). Mercury resistance genes have been detected in bacteria cultured from all of the samples used in this study (data not shown) and may reflect the current limit of detection of the amplification protocol. The *mer* probe hybridized to the 1-kb Fiddler's Ferry and Tn*501* PCR products (Fig. 2b, lanes 3, 4, and 6) but neither to the 800-bp product (Fig. 2b, lane 8) nor to nonamplified DNA (lane 1). Partial sequence analysis of the 800-bp product showed that this is a variant of the Tn*501* sequence.

Taq DNA polymerase has been shown not to be inhibited by substances, such as humic acids, present in DNA extracted from soils amended with target sequences (16, 19, 20, 24). It is only from these amended soils and sediments that successful DNA amplifications have been reported (16, 20, 24); however, our attempts to amplify native bacterial DNA by modifications of these other methods were unsuccessful (data not shown). This is probably a consequence of the impurity of the resultant DNA extracts. Selenska and Klingmüller had previously used their DNA extraction method to illustrate direct DNA hybridization of seeded target sequences from soil without PCR amplification (19). The present study has shown that a modified method can be combined with strict PCR conditions to amplify bacterial genes native to soils and sediments without amendment. The technique was reproducible and detected rRNA genes in high dilutions of the isolated soil DNA. In addition to chromosomal genes (rDNA), the amplification of *mer* genes suggests that plasmid-borne genes can also be detected. This method should assist studies of soil microbiology, microbial ecology, and gene transfer in natural environments, where it is necessary to avoid cultivation of bacteria prior to analysis of gene sequences. Studies are in progress to quantify the bacterial genes present in different soil samples.

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