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Rapid Method for Processing Soil Samples for Polymerase Chain Reaction Amplification of Specific Gene Sequences†

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Bacterial cells can be differentially separated from soil colloids on the basis of their buoyant densities. By using this principle, a modified sucrose gradient centrifugation protocol has been developed for separating bacterial cells from most of the soil colloids. Since the bacterial cell suspension still contained some colloidal soil particles, which inhibited polymerase chain reaction amplification, a new "double" polymerase chain reaction method of analysis was adopted for amplification of Tn5-specific gene sequences. This new protocol allowed rapid detection of small numbers (1 to 10 CFU/g) of bacterial cells present in soil samples.

Polymerase chain reaction (PCR) amplifications have found increasing applications in environmental microbiology (2, 3, 7, 9, 15, 16). Oligonucleotide primer sequences are available for the detection of specific bacterial genera and species (2, 3). The significant advantage of this technique is the potential for detecting very small numbers of target organisms from environmental samples. Since this technique does not involve the culturing of cells, the detection of nonculturable organisms is possible. In particular, PCR amplifications have the potential for monitoring pathogens and indicator bacteria in the environment, especially soils (7).

One of the current limitations of amplifying target sequences from soil samples is the extended processing time involved in separating the cells or DNA species from soil colloids. Holben et al. (6), using cell lysis and differential centrifugation, lysed cells in the soils and subsequently purified the extracted DNA from the soil colloids. Steffan and Atlas (15), citing the need for separating the cells from colloids initially and then extracting the DNA, demonstrated a procedure that involved obtaining DNA from intact cells. This procedure, which has the possibility of excluding "free" DNA adsorbed strongly to soil colloids (10), is time-consuming and labor intensive. Khanna and Stotzky (8) have recently shown that adsorbed DNA does have an ecological significance and can act as a transforming agent. Therefore, it is essential to develop methods that would reduce the chance of excluding ecologically significant adsorbed DNA and would also be efficient and rapid.

In this paper we report the development of a simple method for efficient separation of bacterial cells from most of the soil colloids. Target Tn*5* DNA (*nptII*) sequences in the presence of soil colloids have been amplified by using this extraction method. Research with transposon Tn*5* has shown that it possesses features that make it useful to serve as a biomarker for genetically engineered microorganisms (5, 11). Such biomarkers can be used for labeling genetically engineered microorganisms to study their ecology in the environment. In addition, Tn*5* sequences are unlikely to be found within the indigenous soil population.

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MATERIALS AND METHODS

Soils. Two soils of different textures, Pima clay loam and Brazito sandy loam, were used. Surface samples (depth, 0 to 15 cm) were collected, sieved (mesh size, 2 mm), and stored under field-moist conditions at 4°C. The clay loam had a pH of 8.1, an EC_e (electrical conductivity) of 1.6 mmho/cm, a total N content of 0.05%, and an organic C content of 0.4%. Corresponding values for the sandy loam were pH 8.0, 0.82 mmho/cm, 0.03%, and 0.30%.

Bacterial culture. A transposon Tn*5* insertion mutant of *Rhizobium leguminosarum* bv. phaseoli UAZ 302::Tn*5* (11, 13) was used as the bacterial culture in a determination of the efficiency of extraction and target DNA amplification. This Tn*5* insertion mutant was shown to harbor only a single insertion of the transposable element (4, 11). The inoculum was cultured to the late log phase in peptone yeast extract (PY) medium containing kanamycin and neomycin (30 μg/μl each). The transposable element Tn*5* contains the gene *nptII*, which confers resistance to kanamycin and neomycin (5, 12). The following bacterial cultures, obtained from the culture collection of the Department of Microbiology and Immunology were used for specificity studies: *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Proteus vulgaris*, and *Enterobacter* sp.

Soil inoculation. Subsamples (1 g) of Pima clay loam and Brazito sandy loam were seeded with 0.5-ml decimal dilutions of the bacterial culture which originally contained 6×10^8 and 6×10^7 CFU/ml, respectively. The inoculated soil samples were incubated for 1 h at room temperature to equilibrate and allow for binding of the bacterial cells to the soil colloids.

Extraction of cells. A 9-ml volume of 1% $CaCl_2$ was added to soil subsamples containing variable numbers of cells. The soil-$CaCl_2$ slurry was vigorously vortexed for 1 min and then allowed to settle for 1 h. The upper fraction was concentrated to 5 ml by centrifugation (7,650 × *g* for 5 min) and pipetted into a 50-ml Falcon polypropylene centrifuge tube (Becton Dickinson, Paramus, N.J.) containing 10 ml of sucrose (1.33 g/ml). This soil-cell-sucrose slurry was vigorously vortexed for 1 min. Then 15 ml of sucrose (1.33 g/ml) was carefully layered underneath the slurry. This biphasic gradient was centrifuged at 750 × *g* for 10 min in a Beckman J-6B centrifuge. The clear dilute-sucrose upper fraction (ca. 12 ml) containing the bacterial cells was pipetted into a 40-ml polyallomer centrifuge tube, diluted with water, and centri-

fuged at 12,000 × *g* for 20 min at 10°C in an SS-34 rotor. The bacterial cells, along with soil colloids that had a similar density to the cells, appeared as a pellet. This pellet was directly used as the template for the PCR amplifications.

PCR amplifications. The pellet obtained after the extraction process contained bacterial cells, soil colloids, and perhaps free naked DNA adsorbed to soil colloids. The pellet was resuspended in 30 μl of H_2O and added to the PCR reaction mix containing the reaction buffer, nucleotides (Perkin Elmer Cetus, Norwalk, Conn.), and primers. Primer 1 was [bp 1802] CAC TGA AGC GGG AAG GGA CT; primer 2 was [bp 2101] AGA TCC TCG CCG TCG GGC AT. The composition of the reaction mixture was the same as that described earlier (6) except that the Mg^{2+} concentration was increased to 3.5 mM and 0.4 μg of each primer was used.

The reaction mix was heated to 98°C in the DNA Thermal Cycler (Perkin-Elmer Cetus) for 10 min to lyse the cells. It was then cooled to room temperature; during this time, primers anneal to their target sequences (14). AmpliTaq DNA polymerase (Perkin-Elmer Cetus) was later added, and 25 cycles of PCR were performed by using the Thermal Cycler. At the end of 25 cycles, 10 μl of the amplified product was added to a fresh reaction mix and further amplified for 25 cycles, resulting in a "double-PCR" amplification. The amplified products were detected by using agarose gel electrophoresis and a *nptII*-specific gene probe.

Detection of amplified products. (i) Agarose gel electrophoresis. A 10-μl sample of the amplified product was loaded along with the loading dye onto a 1.6% agarose gel and electrophoresed in 1× TBE buffer (0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA [pH 8.0]) at 5 to 10 V/cm, by using the HE33 unit (Hoefer Scientific Instruments, San Francisco, Calif.). On completion of the electrophoresis, the gel was stained with 1 μg of ethidium bromide per ml and viewed by using a UV transilluminator.

(ii) Gene-specific probe analysis. To determine the sensitivity of the extraction procedure, 5-μl aliquots of the amplified products were dot blotted on GeneScreen Plus (Du Pont, Boston, Mass.) hybridization membrane by using a filtration manifold (Schleicher & Schuell, Keene, N.H.).

The *nptII*-specific gene probe was a 20-bp sequence internal to the 300-bp amplified region (5′-GGCTGATGCAATGC GGCGGC-3′). The oligonucleotide was synthesized by using a 380 A DNA synthesizer (Applied Biosystems, Foster City, Calif.).

The filters were prehybridized for a minimum of 3 h at 50°C in prehybridization buffer (1 M NaCl, 1% sodium dodecyl sulfate [SDS], 10% dextran sulfate, 100 μg of salmon sperm DNA per ml). The probe was prepared by using [γ-^{32}P]ATP (3,000 Ci/mmol) and the forward reaction of T4 polynucleotide kinase (1). Hybridization was carried out overnight at 50°C in a hybridization incubator (Robbins Scientific, Mountain View, Calif.). The filters were washed once for 5 min in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at room temperature and once for 30 min in 2× SSC–1% SDS at 50°C. The filters were later exposed to Kodak XAR-5 film for up to 72 h.

RESULTS AND DISCUSSION

Amplification conditions specific for the *nptII* region of the Tn*5* sequence were optimized. The high G+C content of both the primers and the target sequence (60%) allowed the use of stringent primer-annealing conditions. Figure 1 illustrates the 300-bp Tn*5* sequence along with λ size markers

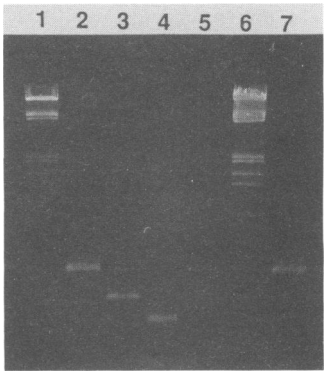

FIG. 1. Agarose gel electrophoresis of PCR-amplified products. Lanes: 1 and 6, *Eco*RI- and *Hin*dIII-digested phase λ; 2 and 7, 500-bp λ fragment (Gene Amp Reagent Kit; Perkin-Elmer Cetus); 3, 300-bp Tn*5* fragment; 4, 179-bp *lamB* fragment (7).

and different-sized fragments. Southern blot analysis confirmed the authenticity of the Tn*5* amplification band (data not included).

Figure 2 shows the specificity of the chosen Tn*5* primer sequences. The primers were tested against DNA preparations from various gram-positive and gram-negative bacterial cultures. Only the Tn*5*-containing cultures showed any specific amplification with the Tn*5* primers. The bacterial genera tested included common soil bacteria and also the wild-type *Rhizobium* sp. from which the Tn*5* insertion mutant was derived (Fig. 2, lane 9). Specific 300-bp amplification products were obtained when the primers were tested against the Tn*5* insertion mutant UAZ 302::Tn*5* and a plasmid preparation (pRZ 102::Tn*5*) containing the Tn*5* sequences (lanes 7 and 8). The difference in intensities of the band is probably due to the difference in amplification efficiency as a result of the location of the target sequence on a plasmid rather than on the main genome. Gene-specific probe hybridization also confirmed the specificity of the primers (data not included).

Figures 3 and 4 demonstrate amplification of Tn*5* se-

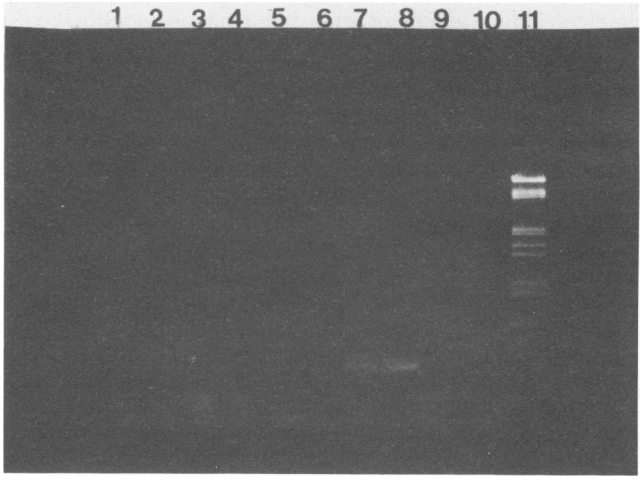

FIG. 2. Specificity of Tn*5* primers. Lanes: 1, *P. aeruginosa*; 2, *A. faecalis*; 3, *K. pneumoniae*; 4, *E. coli*; 5, *Proteus vulgaris*; 6, *Enterobacter* sp.; 7, *R. phaseoli* UAZ 302::Tn*5*; 8, plasmid pRZ102::Tn*5*; 9, *R. phaseoli* CE 3; 10, negative control (no DNA); 11, *Eco*RI- and *Hin*dIII-digested λ.

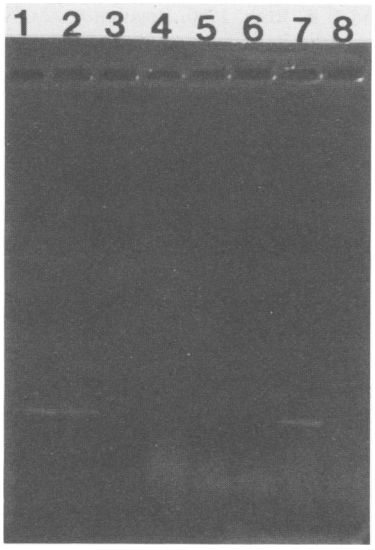

FIG. 3. Sensitivity of amplification of Tn*5* sequences from Pima clay loam: detection by agarose gel electrophoresis. Lanes: 1, 3×10^8 CFU; 2, 3×10^7 CFU; 3, 3×10^6 CFU; 4, 3×10^5 CFU; 5, 3×10^4 CFU; 6, 3×10^3 CFU; 7, positive control (UAZ 302::Tn*5* DNA); 8, uninoculated soil.

quences from soil colloids as detected by ethidium bromide staining. In the clay loam (Fig. 3), detectable amplification of the 300-bp sequences was seen when the soil initially contained a minimum of 10^7 CFU. This is in contrast to the sandy loam, in which 10^6 CFU were detectable (Fig. 4). This difference in sensitivity can be correlated with the differences in the particle size distribution and the cation exchange capacity of the two soil types. The sandy loam could have released cells more easily than the clay loam, and this would subsequently be reflected in a distinct amplification

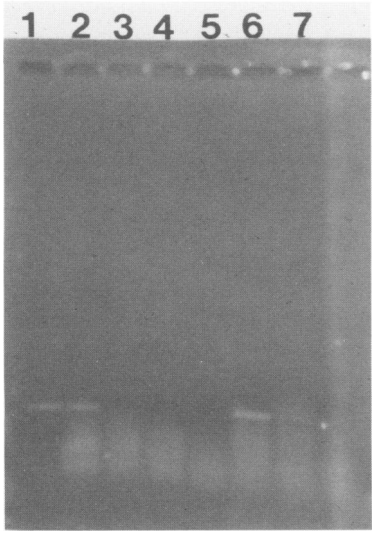

FIG. 4. Sensitivity of amplification of Tn*5* sequences from Brazito sandy loam: detection by agarose gel electrophoresis. Lanes: 1, 3×10^7 CFU; 2, 3×10^6 CFU; 3, 3×10^5 CFU; 4, 3×10^4 CFU; 5, 3×10^3 CFU; 6, positive control (UAZ 302::Tn*5* DNA); 7, uninoculated soil.

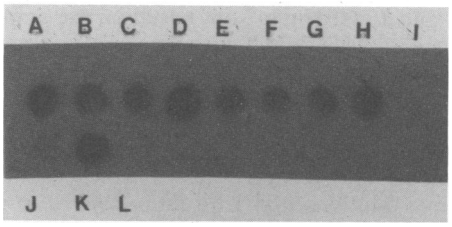

FIG. 5. Sensitivity of amplification of Tn*5* sequences from Pima clay loam: detection by the *nptII*-specific probe. A, 3×10^8 CFU; B, 3×10^7 CFU; C, 3×10^6 CFU; D, 3×10^5 CFU; E, 3×10^4 CFU; F, 3×10^3 CFU; G, 3×10^2 CFU; H, 3×10^1 CFU; I, 3×10^0 CFU; J, negative control (no DNA); K, positive control (UAZ 302::Tn*5* DNA); L, uninoculated soil.

product. The clay loam, which has a higher cation exchange capacity, could have adsorbed the cells more tightly than the sandy loam, thus resulting in reduced cell release. It should be noted, however, that ethidium bromide visualization of PCR-amplified products is not as sensitive as using gene-specific probes to detect amplification products. Since the amplification involved two sets of 25 cycles each, this resulted in nonspecific primer dimer artifacts which are discernible toward the bottom of the agarose gel.

Gene probes specific to an internal 20-bp region of the 300-bp amplified *nptII* region were also used to confirm the sensitivity of the extraction and amplification procedures (Fig. 5 and 6). Figures 5 and 6 show the dot blot of the PCR products after extraction and amplification from the Pima clay loam and the Brazito sandy loam. As few as 1 to 10 CFU were detectable as a positive signal. In the sandy loam soil less than 1 CFU was detectable, most probably because of the release of extra copies of target sequences (due to dead or lysed cells) from this coarser soil. Neither of the negative controls (the uninoculated soil sample and a no-DNA PCR reaction mix) produced a signal. These sensitive detection limits are in contrast to the detection limit obtained with ethidium bromide staining. Moreover, the use of gene-specific probes allows us to differentiate between specific and nonspecific amplification products, which normally occur at the end of a double-PCR amplification. There is no apparent dose dependency based on the amount target sequences available. Under the conditions used, an amplification plateau had probably been reached as a result of depletion of primers or nucleotides or enzyme inactivation. Dose dependency may be evident under reduced amplification cycles.

This method demonstrates the sensitivity of detecting

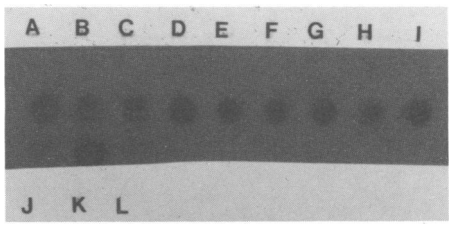

FIG. 6. Sensitivity of amplification of Tn*5* sequences from Brazito sandy loam: detection by the *nptII*-specific probe. A, 3×10^7 CFU; B, 3×10^6 CFU; C, 3×10^5 CFU; D, 3×10^4 CFU; E, 3×10^3 CFU; F, 3×10^2 CFU; G, 3×10^1 CFU; H, 3×10^0 CFU; I, 3×10^{-1} CFU; J, negative control (no DNA); K, positive control (UAZ 302::Tn*5* DNA); L, uninoculated soil.

between 1 and 10 CFU/g of soil, based on the amplification of a single copy of the target sequence contained within the cell (4, 13). This is in contrast to the 15 to 20 copies per cell target sequence used by Steffan and Atlas (15) to demonstrate their extraction methodology. It should be noted that correlating the sensitivity of amplification on the basis of either direct counts or plate counts is fraught with danger, since decimal dilutions that are routinely used to inoculate soil samples for such studies contain, in addition to intact cells, DNA from lysed cells which can serve as targets for PCR amplification (Fig. 6, lane 1).

This method has proved to be a simple, quick, and reliable alternative to the current methods of bacterial-cell extraction from soils. This procedure avoids the labor-intensive and time-consuming published methods. It can be used to extract bacteria from a variety of soils since setting times and centrifugation speeds can be adjusted to achieve optimum layering of bacterial cells in the upper fraction. The only apparent drawback of this technique is the additional requirement of PCR reagents since it involves a double-PCR amplification. This can, however, be overcome by scaling down the reaction volumes to achieve efficient use of reagents.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingdom, D. D. Moore, J. A. Smith, S. G. Sideman, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Bej, A. K., R. J. Steffan, J. Dicesare, L. Haff, and R. M. Atlas.** 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Appl. Environ. Microbiol. **56**:307–314.
3. **Bessesen, M. T., Q. Luo, H. A. Rotbart, M. J. Blaser, and R. T. Ellison III.** 1990. Detection of *Listeria monocytogenes* by using the polymerase chain reaction. Appl. Environ. Microbiol. **56**: 2930–2932.
4. **Biek, D., and J. R. Roth.** 1980. Regulation of of *Tn5* transposition in *Salmonella typhimurium*. Proc. Natl. Acad. Sci. USA **77**:6047–6051.
5. **Frederickson, J. K., D. F. Bezdicek, F. E. Brockman, and S. W. Li.** 1988. Enumeration of Tn*5* mutant bacteria in soil by most-probable-number DNA hybridization procedure and antibiotic resistance. Appl. Environ. Microbiol. **54**:446–453.
6. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
7. **Josephson, K. L., S. D. Pillai, J. Way, C. P. Gerba, and I. L. Pepper.** Detection of fecal coliforms in soil by polymerase chain reaction and DNA-DNA hybridizations. Soil Sci. Soc. Am. J., in press.
8. **Khanna, M., and G. Stotzky.** 1990. Transformation by DNA bound on clay minerals: implications for gene transfer in soil and other habitats, abstr. Q-191, p. 320. Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990. American Society for Microbiology, Washington, D.C.
9. **Mahubani, M. H., A. K. Bej, R. Miller, L. Haff, J. Dicesare, and R. M. Atlas.** 1991. Detection of *Legionella* by using polymerase chain reaction and gene probe methods. Mol. Cell. Probes **4**:175–187.
10. **Ogram, A., G. S. Sayler, D. Gustin, and R. L. Lewis.** 1988. DNA adsorption to soils and sediments. Environ. Sci. Technol. **22**:982–984.
11. **Pillai, S. D.** 1989. Ph.D. thesis. University of Arizona, Tucson.
12. **Pillai, S. D., and I. L. Pepper.** 1990. Survival of *Tn5* mutant bean rhizobia in desert soils: phenotypic expression of *Tn5* under moisture stress. Soil Biol. Biochem. **22**:265–270.
13. **Pillai, S. D., and I. L. Pepper.** 1991. Transposon *Tn5* as an identifiable marker in rhizobia: survival and genetic stability of *Tn5* mutant bean rhizobia under temperature stressed conditions in desert soils. Microb. Ecol. **21**:21–33.
14. **Sandhu, G. S., J. W. Precup, and B. C. Kline.** 1989. Rapid one-step characterization of recombinant vectors by direct analysis of transformed *Escherichia coli* colonies. Biotechniques **7**:689–690.
15. **Steffan, R. J., and R. M. Atlas.** 1988. DNA amplification to enhance detection of genetically engineered bacteria in environmental samples. Appl. Environ. Microbiol. **54**:2185–2191.
16. **Way, J. S., K. L. Josephson, S. D. Pillai, C. P. Gerba, and I. L. Pepper.** Unpublished data.