DNA Amplification To Enhance Detection of Genetically Engineered Bacteria in Environmental Samples

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The polymerase chain reaction (PCR) was performed to amplify a 1.0-kilobase (kb) probe-specific region of DNA from the herbicide-degrading bacterium *Pseudomonas cepacia* AC1100 in order to increase the sensitivity of detecting the organism by dot-blot analysis. The 1.0-kb region was an integral portion of a larger 1.3-kb repeat sequence which is present as 15 to 20 copies on the *P. cepacia* AC1100 genome. PCR was performed by melting the target DNA, annealing 24-base oligonucleotide primers to unique sequences flanking the 1.0-kb region, and performing extension reactions with DNA polymerase. After extension, the DNA was again melted, and the procedure was repeated for a total of 25 to 30 cycles. After amplification the reaction mixture was transferred to nylon filters and hybridized against radiolabeled 1.0-kb fragment probe DNA. Amplified target DNA was detectable in samples initially containing as little as 0.3 pg of target. The addition of 20 μg of nonspecific DNA isolated from sediment samples did not hinder amplification or detection of the target DNA. The detection of 0.3 pg of target DNA was at least a 10^3-fold increase in the sensitivity of detecting gene sequences compared with dot-blot analysis of nonamplified samples. PCR performed after bacterial DNA was isolated from sediment samples permitted the detection of as few as 100 cells of *P. cepacia* AC1100 per 100 g of sediment sample against a background of 10^{11} diverse nontarget organisms; that is, *P. cepacia* AC1100 was positively detected at a concentration of 1 cell per g of sediment. This represented a 10^3-fold increase in sensitivity compared with nonamplified samples.

The recent interest in the possibility of intentionally introducing genetically engineered microorganisms into the environment (7, 14, 22) has led us and others to try to develop sensitive methods for detecting specific genetically defined microorganisms within the complex microbial communities of natural ecosystems (2, 11, 12, 16, 26, 28, 36). Methods for measuring the abundance of a particular organism based on culturing the organism on either general-purpose or selective medium and screening colonies for the presence of a specific phenotype have been supplemented by the development of colony hybridization methods that allow the detection of a specific genotype (3, 4, 13, 24, 28). These colony hybridization methods still depend on the ability to recover and culture the organism from an environmental sample, as do the classic plating procedures based on recognition of specific phenotypes.

Several reports have addressed the difficulty and limited efficiency of recovering bacteria from natural environments by methods that depend on culturing those microbes (1, 29). Some recent studies have focused on methods of detecting microorganisms in environmental samples that do not require direct culturing of the organism. In these methods biochemical constituents of microbial cells are recovered and analyzed either for specific phenotypic markers (6, 35) or for genotypic markers (16, 25; R. J. Steffan and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, Q130, p. 303). In one such approach, bacterial cells are removed from the environmental sample, and their DNA is recovered and purified (16; 34; R. J. Steffan, J. Goksøyr, and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, Q144, p. 308). The DNA is then spotted onto filters, and the presence or absence of a given genotype is determined by hybridization to a radiolabeled gene probe (25). Although these methods may be more sensitive than plating methods,

they still may lack the sensitivity required to determine the ultimate fate of introduced organisms because of the limited relative numbers of target genetic sequences that may be present in the sample.

The newly developed polymerase chain reaction (PCR) provides a method for increasing the number of copies of a target sequence (amplifying the signal) without having to culture the organism, thereby allowing increased sensitivity in detecting DNA sequences present in small amounts in samples with DNA from mixed populations (27, 30, 31, 33). The method involves melting the DNA and annealing short oligomer primers to regions flanking a target sequence. DNA polymerase is added to the mixture in the presence of free deoxynucleotides, and DNA is extended from the primers across the target region. The new duplexes are again melted and the process is repeated. This results in the exponential accumulation of the specific target, approximately 2^n, where n is the number of cycles of melting and primer extension. Single-copy genomic sequences can be amplified by a factor of more than 10 million with high specificity (30, 33). The method has been refined by the use of a thermally stable polymerase isolated from *Thermus aquaticus* (*Taq*), which obviates the need to add new polymerase after each melting cycle, and has been shown to have great specificity (30).

PCR with *Taq* polymerase has been used successfully to amplify human β-globin genes (30) and to detect human immunodeficiency virus type 1 (HIV-1) proviral DNA from human cells (27). However, PCR has not previously been used with environmental samples. In this study we examined the ability of the PCR method to amplify a specific repeated target sequence from the "genetically engineered" herbicide-degrading bacterium *Pseudomonas cepacia* AC1100 after this organism had been introduced at various concentrations into environmental samples with large mixed microbial populations.

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MATERIALS AND METHODS

Sediment collection and characterization. Surficial sediments were collected from the Ohio River at Louisville, Ky., with an Ekman dredge. Sediment samples were pooled, mixed, and maintained at 5°C. Analyses were initiated within a few hours of sample collection. Dry-weight analysis was performed by drying sediments to a constant weight at 105°C. Organic content of dried sediments was determined gravimetrically after combustion at 550°C for 1 h. Physical composition (i.e., percent clay, silt, and sand) was determined as described in Black (5). Bacterial numbers in the sediment were determined by acridine orange direct count (AODC) (15) and by viable plating onto one-tenth-strength Trypticase soy agar (BBL Microbiology Systems). Sediment characteristics were: dry weight, 52%; organic content, 12%; sand, 55%; silt, 30%; clay, 15%; viable heterotrophs, 4.6×10^6 CFU/g (wet weight); total cells (AODC), 2.3×10^9/g (wet weight).

Addition of *P. cepacia* AC1100 to sediments. *P. cepacia* AC1100 was grown to early log phase in basal salts medium (17) containing 1.0 mg of 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) per ml. Cell density was determined by absorbance at 540 nm as described previously (20). Plate counts on Trypticase soy agar were performed so that the exact number of cells actually added to each sample could later be specified. The *P. cepacia* AC1100 culture was diluted in 0.1 M sodium phosphate buffer (pH 6.8) and added to duplicate 100-g sediment samples in a volume of 10 ml to give final concentrations of approximately 10^4, 10^2, 10^0, and 10^{-1} cells per g. Control samples with no added cells of *P. cepacia* AC1100 were also included. The samples were thoroughly mixed by vigorous swirling. The sediments were then incubated at 28°C for 30 min to allow some binding to sediment.

Extraction of bacterial cells from sediments. The bacterial fraction of the sediments, including the added cells of *P. cepacia*, was separated from the inorganic fraction by using a modification of a method previously developed for recovery of bacteria from soils for genetic analyses (10, 34; R. J. Steffan, J. Goksoyr, and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, Q144, p. 308); the method is based on successive cycles of blending and differential centrifugation to recover the intact bacterial cells, followed by cell lysis and recovery and purification of the bacterial DNA.

One hundred grams of sediment was suspended in 300 ml of 0.1 M sodium phosphate buffer (pH 4.5) and homogenized in a Waring blender. Samples were blended at medium speed three times for 1 min each, with 1 min of cooling on ice between each blending cycle. Two milliliters of 20% sodium dodecyl sulfate (SDS) was added to each sample, and the samples were blended for an additional 5 s. The samples were placed on ice, the foaming was allowed to settle for 5 min, and the samples were then transferred to 250-ml centrifuge bottles. The bottles were shaken by hand for 1 min and centrifuged in a GSA rotor (Sorvall RS-5 centrifuge) for 10 min at $1,000 \times g$ and 10°C. The supernatants from each individual replicate were pooled in an Erlenmeyer flask and maintained on ice until further centrifugation.

The sediment pellets were washed back into the blender with 300 ml of 0.1 M sodium phosphate buffer (pH 4.5) and further blended and centrifuged as described above, but without further addition of SDS. The supernatants were combined with the earlier pooled supernatants and maintained on ice. The combined supernatants were centrifuged for 30 min at $10,000 \times g$ and 10°C to collect the bacterial

cells. The sedimented material containing the combined bacterial cell fraction was suspended in a 200 ml of 0.1% sodium hexaphosphate–0.1% sodium pyrophosphate at 5°C. The samples were shaken by hand for 1 min and then centrifuged for 30 min at $10,000 \times g$ and 10°C. The supernatant was discarded and the procedure was repeated. These washings decreased the amount of particulate organic (nonbacterial) material in the cell pellet.

As a final washing procedure, the cell pellet was suspended in 150 ml of Chrombach buffer (0.33 M Tris hydrochloride, 0.001 M EDTA, pH 8.0) and centrifuged as described for previous washings. This final wash appeared to remove a large portion of the remaining humic material in the cell pellet. The final pellet was transferred to a 50-ml centrifuge tube by washing with Chrombach buffer and adjusted to a final volume of 25 ml.

Cell lysis and DNA purification. The cell pellets were mixed vigorously on a Vortex mixer to completely suspend any clumps of sedimented material. Lysozyme (Sigma Chemical Co.) was added to final concentrations of 5 mg/ml, and the suspensions were incubated for 2 h at 37°C. The suspensions were then heated to 60°C, and SDS was added to a final concentration of 1.0%, after which they were incubated for 10 min. The suspensions were cooled on ice for 2 h and then centrifuged for 20 min at $12,000 \times g$ in a Sorval SS34 rotor at 5°C. The supernatant (first lysate) was transferred to a sterile centrifuge tube. The pelleted material was washed with 10 ml of Chrombach buffer and centrifuged as described above. The supernatant solution was collected and combined with the first lysate.

DNA purification. To purify the DNA in the combined cell lysate, solid ammonium acetate was added to the samples to give a final concentration of 2.5 M and the samples were immediately centrifuged for 30 min at $12,000 \times g$ and 5°C. This process resulted in the precipitation of a considerable amount of organic debris, but no DNA could be detected in this precipitated material by cesium chloride-ethidium bromide density gradient centrifugation.

DNA in the supernatant fraction was precipitated by addition of 2.5 volumes of ice-cold 95% ethanol and incubation at −70°C for 1 h. The samples were centrifuged for 30 min at $12,000 \times g$ and 5°C, the ethanol was decanted, and the pellets were dried under vacuum. The DNA was suspended in 10 ml of TE (10 mM Tris hydrochloride, 1 mM EDTA, pH 8.0) and separated by cesium chloride-ethidium bromide density gradient centrifugation (23). The resultant DNA band was collected, ethidium bromide was removed by successive extractions with water-saturated 1-butanol, and the samples were dialyzed for 24 h against two changes of TE. The DNA was precipitated with ethanol as above and suspended in 100 μl of sterile double-distilled water. Subsamples of the recovered DNA sample were analyzed by agarose gel electrophoresis (Fig. 1), and the purity of the DNA was checked by spectrophotometric absorbancy measurements at 280 and 260 nm.

PCR amplification of RS-1100-I. *P. cepacia* AC1100 contains a highly repeated 1.3-kilobase (kb) sequence (15 to 20 copies per cell), designated RS-1100-1, that occurs both on the chromosome and in its heterogeneous plasmid population (P. Tomasek and A. Chakrabarty, personal communication). The entire nucleotide sequence of this 1.3-kb region has been determined (P. Tomasek and A. Chakrabarty, personal communication). PCR amplification of the RS-1100-I sequence was performed by using *Thermus aquaticus* (*Taq*) DNA polymerase and a GeneAmp kit (Cetus Corp., Emeryville, Calif.) as described by Saiki et al. (30). PCR conditions
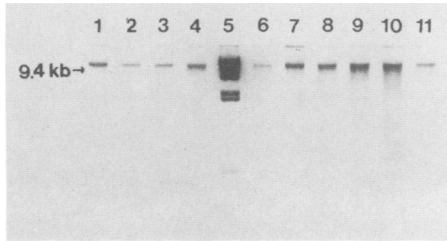
FIG. 1. Electrophoresis of DNA isolated from sediments amended with *P. cepacia* AC1100 on a 0.7% agarose horizontal gel. Each sample represents approximately 1/20 of the entire DNA sample recovered from the sediment. Lanes: 1 and 2, no added *P. cepacia* AC1100; 3 and 4, 10^{-1} cells added per g; 5, 1.2 μg of lambda DNA digested with *Hind*III; 6 and 7, 10^0 cells added per g; 8 and 9, 10^2 cells added per g; 10 and 11, 10^4 cells added per g. The replicate containing the least amount of DNA was selected for PCR.

were optomized by several trial extensions with pRS19U-5 containing the RS-1100-I fragment. These preliminary tests involved the use of several different primers and alteration of the reaction temperatures and incubation times. Primers finally selected for use in amplification experiments consisted of 24-base oligomers specific for sites flanking the unique *Bss*HII (primer RSB02) and *Xho*I (primer RSX01) restriction enzyme recognition sites of RS-1100-I (Fig. 2). Computer analysis of the entire RS-1100-I sequence indicated that the first 5-base sequence on the 3' end of each primer was unique within the RS-1100-I fragment, making the primers more specific than the required minimal first 6-base sequence on the 3' end needed to ensure production of full-length transcripts (Stephen J. Scharf, Cetus Corp., personal communication). Incubation times were increased from the manufacturer's recommendations to 6 min at 70°C for extension, 3 min at 94°C for melting, and 2 min at 40°C for primer annealing, to ensure that optimum temperatures were achieved within the reaction mixture.

Two separate experiments were conducted to determine the ability to perform PCR in the presence of nonspecific DNA from the mixed populations of environmental communities. In the first experiment, pRS19U-5 DNA (pTZ19U; U.S. Biochemical Co., 2.8 kb, into which the RS-1100-I sequence had been cloned to produce a 4.1-kb plasmid maintained in *Escherichia coli*) was diluted in 10 μl of double-distilled water in duplicate to give final amounts of

target sequence in the range of 0.3 ng to 0.3 pg. Twenty micrograms of nonspecific DNA isolated from sediments not amended with *P. cepacia* AC1100 was added to one of the replicates at each target sequence concentration, and 25 cycles of PRC were performed as described below. Controls were prepared as above and not subjected to PCR.

In the second set of experiments, PCR was performed with the DNA recovered from sediments to which various concentrations of *P. cepacia* AC1100 had been added. The amount of DNA was quantitated by absorbance measurement at 260 nm, and the replicate sample from each concentration of added *P. cepacia* AC1100 containing the least amount of DNA was selected for amplification to ensure that any differences observed between the amplified and nonamplified samples were due to target sequence amplification and not to extraction effiency. Thirty cycles of PCR were used. The other paired replicate was used as a comparative nonamplified control.

For PCRs, the sediment DNA was precipitated with ethanol and suspended in the reaction mixture as described below. PCRs were performed in sterile siliconized 1.5-ml microfuge tubes in a volume of 100 μl containing the DNA (30 to 90 μg of extracted sediment DNA or 1.0 pg to 1 ng of pRS19U-5 DNA with or without the addition of 20 μg of sediment DNA) in 50 mM KCl–10 mM Tris (pH 8.4)–2.5 mM $MgCl_2$–0.6 μg of each primer (RSX01 and RSB02)–250 μM each dATP, dCTP, dGTP, and dTTP–200 μg of gelatin per ml. Samples were heated to boiling for 5 min to destroy any proteinase activity in the DNA preparation. The samples were then cooled in a water bath to 40°C, and 2.5 U of *Taq* polymerase was added. The samples were overlaid with 100 μl of sterile mineral oil to prevent evaporation and subjected to repetitive cycles of amplification as follows. After 1 min at 40°C, they were transferred to a 70°C water bath for extension, incubated for 6 min, and then transferred to a dry-heat block at 94°C to melt the DNA and incubated for 3 min. Subsequent cycles were 2 min at 40°C, 6 min at 70°C, and 3 min at 94°C. At the end of 30 cycles, the samples were incubated at 70°C for an additional 7 min to ensure that the final extension was complete.

Probes and DNA hybridization. Following completion of the amplification procedure, the samples were allowed to equilibrate to room temperature and transferred directly to nylon membrane filters (Biotrans; 0.2-μm pore size; ICN Biochemicals, Inc., Irvine, Calif.) by using a Bio-Dot appa-
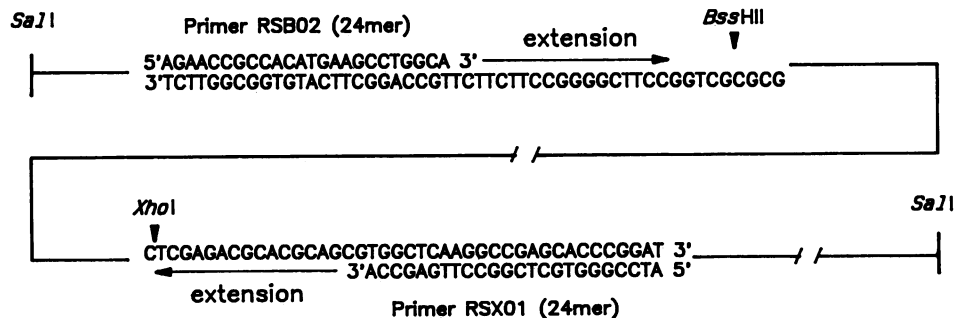
RS—1100—1 fragment

FIG. 2. Schematic representation of primer positioning on the RS-1100-I fragment for performing PCR. The *Bss*HII-*Xho*I restriction fragment was used as a probe throughout this study.

ratus (Bio-Rad Laboratories). After drying, the filters were placed on filter paper pads containing denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 5 min and then on pads containing neutralizing solution (3 M sodium acetate, pH 5.5) for 5 min. The filters were then dried for 30 min at room temperature, and the DNA was bonded to the filters by irradiating them for 2 min under a short-wavelength UV light. Autoradiography was performed as previously described (23).

To establish a probe, the RS-1100-I was isolated and cloned into the *Sal*I site of PS1 EMBL (pPT165; Paul Tomasek, personal communication). The plasmid pRS19U-5 (4.1 kb) was created by subsequently subcloning the RS-1100-I fragment from pPT165 into the *Sal*I site of pTZ19U (2.8 kb; U.S. Biochemical, Cleveland, Ohio). Colony hybridization screening of several hundred Ohio River sediment and water isolates, with radiolabeled RS-1100-I as a gene probe, demonstrated no nonspecific cross-hybridization to the probe. We therefore found it to be a suitable probe for detecting *P. cepacia* AC1100 in this system. For the PCR experiments, a shorter 1,020-base-pair (bp) *Bss*HII-*Xho*I fragment, integral to the RS-1100-I sequence, was used as a probe for detecting the amplified DNA sequence so as to avoid hybridization to the primers (Fig. 2).

The 1,020-bp *Bss*HII-*Xho*I fragment of RS-1100-I was removed from pRS19U-5 by restriction endonuclease digestion. The fragment was isolated by direct extraction (23) after electrophoresis in a 1.0% Sea Plaque agarose gel (FMC Corporation, Rockland, Maine). The probe (1.0 μg) was radiolabeled to a high specific activity with [α-^{32}P]dATP (ICN) by using a nick translation kit (Amersham). Dot-blot filters were incubated for 2 h at 42°C in prehybridization solution containing 5× Denhardt solution (100× Denhardt solution is 2% Ficol 400,000, 2% polyvinylpyrrolidone, 2% bovine serum albumin), 5× SSC (20× SSC is 3 M NaCl, 0.3 M sodium citrate, pH 7.0), 50 mM sodium phosphate (pH 6.5), 0.1% SDS, 250 ng of denatured sonicated salmon sperm DNA per ml, and 50% deionized formamide. The prehybridization solution was then removed and replaced with hybridization solution (same as prehybridization solution but without salmon sperm DNA) and 0.5 μg of the radiolabeled probe (specific activity, 2 × 10^7 cpm/μg) and incubated for 18 h at 42°C. The filters were then washed twice for 30 min each in 2× SSC –0.1% SDS at 55°C and twice for 30 min each in 0.1× SSC–0.1% SDS at 55°C. Autoradiography was performed for 4 h at −70°C as described previously (23).

RESULTS

In the first set of experiments performed to determine the ability to conduct PCR in the presence and absence of DNA isolated from sediments, the RS-1100-I sequence was amplified to detectable levels in samples containing as little as 0.3 pg of target against a background of 20 μg of sediment DNA after 25 cycles of PCR (Fig. 3). No difference was found between samples with and without added nonspecific sediment DNA. This level of detection represented potential detection of as few as 10^4 cells of *P. cepacia* AC1100 against a background of 10^{11} cells, but the actual detection limit of this method was actually lower (see below) because amplified DNA was detected in all samples tested here, so that the real lower limit of detection was not determined. Nonamplified RS-1100-I was only detected in samples containing 0.3 ng of target against the 20-μg background of sediment DNA. Thus, a specific gene sequence amplified by PCR was detectable with three orders of magnitude greater sensitivity than in nonamplified samples.
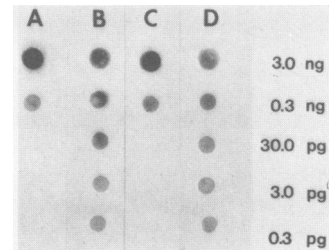

FIG. 3. Dot-blot hybridization analysis of amplified and nonamplified RS-1100-I fragment in the presence and absence of sediment DNA. Values represent the amount of RS-1100-I sequence before amplification. Columns A and C contain the entire sample of nonamplified RS-1100-I in the absence and presence of 20 μg of sediment DNA, respectively. Columns B and D contained one-half of the amplified DNA sample. The RS-1100-I fragments in B and D were amplified to detectable levels after 25 cycles of PCR in the absence and presence of 20 μg of sediment DNA, respectively. All signals detected were significantly above background levels observed with negative controls.

In the second set of experiments involving sediment DNA extraction and PCR, cells of *P. cepacia* AC1100 were added directly to the sediment, and amplified RS-1100-I was detected in samples initially containing only one *P. cepacia* AC1100 cell per g of sediment extracted (Fig. 4). In nonamplified samples the RS-1100-I sequence was detectable in samples containing 10^4 *P. cepacia* AC1100 cells per g. Signals produced from the nonamplified sample containing 10^2 *P. cepacia* AC1100 cells per g were not significantly above background levels and therefore were not recorded as positive detection of the organism. This is consistent with our previous experience with the dot-blot method in environmental samples, in which we have repeatedly found a lower limit of 10^3 cells of *P. cepacia* AC1100 per g of sediment for dot-blot detection of nonamplified extracted DNA (R. J. Steffan and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, Q130, p. 303).

DISCUSSION

P. cepacia AC1100 is a 2,4,5-T-degrading strain that was formed by using plasmid-assisted molecular breeding (18, 19). This genetically engineered bacterium uses 2,4,5,-T as a sole source of carbon and energy in pure culture and is capable of rapidly degrading this herbicide in soils (8, 17, 20, 21). As an example of the limited sensitivity of cultural methods applied to detecting genetically engineered microorganisms in natural ecosystems, when this herbicide-degrading bacterium was added to soil its population was no longer detectable by plating methods shortly thereafter,
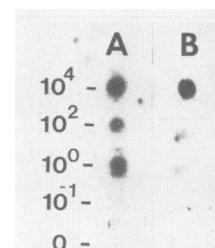

FIG. 4. Dot-blot hybridization analysis for the presence of the RS-1100-I sequence in DNA isolated from 100 g of sediments amended with 10^{-1} to 10^4 cells of *P. cepacia* AC1100 per g. (A) Samples subjected to 30 cycles of PCR; (B) nonamplified samples.

although the organism was still present, as evidenced by its rapid reappearance at detectable levels when 2,4,5-T, a herbicide that appears to be used exclusively by this organism, was added to the soil as an enrichment factor (21).

Several reports have addressed the difficulty of culturing organisms from environmental samples and detecting or enumerating specific organisms (1, 29). The problems arise from the lack of a suitable medium or culture conditions which will allow the growth of all organisms in a particular sample, inefficient recovery of microorganisms bound to particles, and the inability to recover some viable organisms from environmental samples—the so-called viable but nonculturable microorganisms. Although this is a general problem for the detection of microorganisms in environmental samples, the efficiency of detection is especially critical when tracking the fate of genetically engineered microorganisms in the environment.

Some studies have reported low levels of detection of genetically defined microorganisms by selective plating and colony hybridization methods. For example, Sayler et al. (32) reported the ability to detect, by colony hybridization, 1 colony of a specific catabolic genotype in 10^6 colonies of nonhomologous background. The actual number of organisms with the target genotype may have been greater but not culturable on the medium used in the viable plating procedure. Similarly, Devanas et al. (9) reported the ability to detect as few as 1 to 20 organisms containing specific plasmids per g of soil by isolation on selective medium, but here again the actual number of the organisms remaining in the sample could have been significantly greater than the numbers observed afterward by using the viable plating procedure. In fact, their observation that some of the plasmid-containing strains could not be detected on selective medium but could be recovered after transfer from nonselective medium suggests that nonculturable cells were going undetected by the selective viable plating procedure.

The direct DNA extraction–dot-blot detection method, which does not require culturing the target organisms, has an advantage over conventional methods in its potential to detect viable but nonculturable cells. The sensitivity of this method depends on several factors, including DNA extraction efficiency, number of target sequences, specificity and specific activity of probes, and autoradiographic exposure time. Estimates of DNA recovery from soil, sediment, and water samples range from 50 to 90% (16, 25, 34; R. J. Steffan, J. Goksoyr, and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, Q144, p. 308). The cell recovery efficiency for the method used in this study was approximately 40%, as determined by microscopic AODC of the recovered fractions. This is much better than the estimated 1% cell recovery from mixed communities achieved by viable plating.

A major limiting factor in the dot-blot procedure, however, is the limited amount of DNA that can be bonded onto the hybridization filters. This problem can be overcome in part by increasing the relative concentration of target DNA within the total DNA sample. Achieving relatively higher concentrations of target than of nontarget DNA can be accomplished by using target sequences that are maintained on high-copy-number plasmids or that occur as repeated sequences, as is the case with P. cepacia AC1100. Alternatively, enrichment culture procedures can be used to increase the relative concentration of organisms with the target against a background of non-target-containing organisms as demonstrated in the method developed by Fredrick-

son et al. (12); this approach, however, still requires cell growth.

In this study we found that PCR can be used as an in vitro alternative method for rapidly increasing the number of targets that does not require cell growth for the detection of specific genetically defined microorganisms within the complex microbial communities of environmental samples. Use of this method does require the synthesis of oligonucleotide primers, and thus the procedure is limited to cases in which the gene sequence of the target is known. The PCR reaction was successful even though the DNA recovered from the sediments was not highly purified. Some organic material remained associated with the DNA, as indicated by a slight yellow color in some samples, and ratios of spectrophotometric absorbancy at 260 and 280 nm ranged from 1.3 to 1.7. Additional purification could have been achieved by using hydroxyapatite chromatography (10), a procedure we have used previously to purify DNA from environmental samples, or by using polyvinylpolypyrrolidone to remove humic materials (16), but our results indicate that this additional purification is not essential to perform PCR.

We did find it necessary, however, to increase the recommended incubation times to ensure that the optimum temperatures were achieved within the PCR process. This was particularly important in the denaturation steps to allow complete dissociation of newly formed duplexes, which is required for efficient amplification. The theoretical amplification for the reactions performed here were 3.4×10^7 for the reactions with known amounts of pRS19U-5 (25 cycles; Fig. 3) and 1.1×10^9 for the amplification of the DNA recovered from sediments amended with P. cepacia AC1100 (30 cycles; Fig. 4). The actual yields in our experiments were not determined because we were primarily interested in enhancing our ability to detect the target sequence, but it is likely that the amplification efficiency was less than 100% due to the presence of contaminants in the DNA extracted from sediments.

In the initial PCR experiments, in which various amounts of target sequence were amplified, we were readily able to detect 0.3 pg of target after amplification even in the presence of nonspecific DNA isolated from sediments. This represented a 1,000-fold increase in sensitivity compared with nonamplified samples. We should note, however, that in previous experiments with high-specific-activity RNA probes and longer autoradiographic exposures at $-70°C$ we have detected as little as 3 pg of target sequence without amplification (R. J. Steffan and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, Q130, p. 303); even considering this lower detection level for nonamplified samples, the PCR method gave at least a 10-fold increase in the amount of target detected over that with conventional dot-blot methods. This does not indicate a detection limit, however, because target DNA was detected in all of our amplified samples and no lower dilutions were tested in the PCR experiments. Thus, PCR clearly can be used to significantly enhance the detectability of gene sequences within a very heterogeneous DNA pool.

Despite the fact that we were working with DNA from complex microbial communities with very high background numbers of nontarget organisms, the detection level we observed with PCR was similar to that reported by Saiki et al. (30), who were able to detect a β-globin gene which occurs in only 1 copy per 500,000 human cells tested, and Ou et al. (27), who detected HIV-1 proviral DNA genes that occur only once in 10,000 peripheral blood mononuclear cells of infected patients. The ability to detect 1 cell of P.

cepacia AC1100 per g of sediment (100 cells in the 100-g sediment sample extracted) by using PCR, which was demonstrated when target cells were added to sediments, is a significant improvement in the detection limit obtainable by conventional dot-blot methods. This detection level represents approximately 15 copies of the target sequence per g of sediment, or 1,500 copies of the sequence per the total 100-g sediment sample. It is at least three orders of magnitude better than we have attained by using conventional (nonamplified) dot-blot methods, with which we have been able to detect approximately 10^3 cells per g of sediment, and it is as sensitive as any method ever reported for detecting genetically engineered microorganisms in environmental samples. Even greater sensitivity may be obtained by combining PCR with solution hybridization procedures (R. J. Steffan and R. M. Atlas, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, Q130, p. 303). The procedure may also be made quantitative by incorporating PCR into a procedure such as the one described by Frederickson et al. (12).

In conclusion, this study extends the applicability of PCR to environmental samples and significantly enhances the ability to detect genetically defined microorganisms in environmental samples. Development of this method greatly improves our ability to track genetically engineered microbes that are released into the environment.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Atlas, R. M.** 1982. Enumeration and estimation of biomass of microbial components in the biosphere, p. 84–102. *In* R. G. Burns and J. H. Slater (ed.), Experimental microbial ecology. Blackwell Scientific Publishers, Oxford.
2. **Atlas, R. M., and G. S. Sayler.** 1988. Tracking microorganisms and genes in the environment, p. 31–45. *In* G. S. Omenn (ed.), Environmental biotechnology: reducing risks from environmental chemicals through biotechnology. Plenum Press, New York.
3. **Barkay, T., D. L. Fouts, and B. H. Olson.** 1985. The preparation of a DNA gene probe for the detection of mercury resistance genes in gram-negative bacterial communities. Appl. Environ. Microbiol. **49:**686–692.
4. **Barkay, T., and B. H. Olson.** 1986. Phenotypic and genotypic adaptation of aerobic heterotrophic sediment bacterial communities to mercury stress. Appl. Environ. Microbiol. **52:**403–406.
5. **Black, C. A. (ed.).** 1965. Methods of soil analysis. American Society of Agronomy, Madison, Wis.
6. **Bohlool, B. B., and E. L. Schmidt.** 1980. The immunofluorescence approach in microbial ecology. Adv. Microb. Ecol. **4:** 203–241.
7. **Brown, T. H., R. K. Colwell, R. E. Lenski, B. R. Levin, M. Lloyd, P. J. Regal, and D. Simberloff.** 1984. Report on workshop on possible ecological and evolutionary impacts of bioengineered organisms released into the environment. Bull. Ecol. Soc. Am. **65:**436.
8. **Chatterjee, D. K., J. J. Kilbane, and A. M. Chakrabarty.** 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid in soil by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. **44:**514–516.
9. **Devanas, M. A., D. Rafaeli-Eshkol, and G. Stotzky.** 1986. Survival of plasmid-containing strain of *Escherichia coli* in soil: effect of plasmid size and nutrients on survival of hosts and maintenance of plasmids. Curr. Microbiol. **13:**269–277.
10. **Faegri, A., V. L. Torsvik, and J. Goksoyr.** 1977. Bacterial and fungal activities in soil: separation of bacteria by a rapid fractionated centrifugation technique. Soii Biol. Biochem. **9:** 105–112.
11. **Ford, S., and B. H. Olson.** 1988. Methods for detecting genetically engineered microorganisms in the environment. Adv. Microb. Ecol. **10:**45–79.
12. **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 and antibiotic resistance. Appl. Environ. Microbiol. **54:**446–453.
13. **Grunstein, M., and D. S. Hogness.** 1975. Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. USA **72:**3961–3965.
14. **Halvorson, H. O., D. Pramer, and M. Rogul.** 1985. Engineered organisms in the environment: scientific issues. American Society for Microbiology, Washington, D.C.
15. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
16. **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.
17. **Karns, J. S., S. Duttagupta, and A. M. Chakrabarty.** 1983. Regulation of 2,4,5-trichlorophenoxyacetic acid and chlorophenol metabolism in *Pseudomonas cepacia* AC1100. Appl. Environ. Microbiol. **46:**1182–1186.
18. **Karns, J. S., J. J. Kilbane, D. K. Chatterjee, and A. M. Chakrabarty.** 1981. Laboratory breeding of a bacterium for enhanced degradation of 2,4,5-T, p. 37–40. *In* J. Crocomo, F. C. A. Tavares, and D. Sodrzeieski (ed.), Genetic engineering for biotechnology. Promocet, Sao Paulo, Brazil.
19. **Kellogg, S. T., D. K. Chatterjee, and A. M. Chakrabarty.** 1981. Plasmid assisted molecular breeding—new technique for enhanced biodegradation of persistent toxic chemicals. Science **214:**1133–1135.
20. **Kilbane, J. J., D. K. Chatterjee, J. S. Karns, S. T. Kellog, and A. M. Chakrabarty.** 1982. Biodegradation of 2,4,5-trichlorophenoxyacetic acid by a pure culture of *Pseudomonas cepacia*. Appl. Environ. Microbiol. **44:**72–78.
21. **Kilbane, J. J., D. K. Chatterjee, and A. M. Chakrabarty.** 1983. Detoxification of 2,4,5-trichlorophenoxyacetic acid from contaminated soil by *Pseudomonas cepacia*. Appl. Environ. Microbiol. **45:**1697–1700.
22. **Levin, M. A., R. Seidler, A. W. Bourquin, J. W. Fowle III, and T. Barkay.** 1986. EPA developing methods to assess environmental release. Bio/Technology **5:**38–45.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **Moseley, S. L., U. Huq, A. R. M. A. Alim, M. So, M. Samad-pour-Motalebi, and S. Falkow.** 1980. Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. J. Infect. Dis. **142:**892–898.
25. **Ogram, A., G. S. Sayler, and T. Barkay.** 1988. DNA extraction and purification from sediments. J. Microbiol. Methods **7:**57–66.
26. **Omenn, G. S.** 1986. Controlled testing and monitoring methods for microorganisms, p. 144–162. *In* J. Fiksel and V. T. Covello (ed.), Biotechnology risk assessment: issues and methods for environmental introductions. Pergamon Press, New York.
27. **Ou, C. Y., S. Kwok, S. W. Mitchell, D. H. Mack, J. J. Sninsky, J. W. Krebs, P. Feorino, D. Warfield, and G. Schochetman.** 1988. DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science **239:**292–295.
28. **Pettigrew, C., and G. S. Sayler.** 1986. The use of DNA:DNA colony hybridization in the rapid isolation of 4-chlorobiphenyl degradative bacterial phenotypes. J. Microbiol. Methods **5:**205–213.
29. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
30. **Saiki, R. K., D. H. Glenfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich.** 1988. Primer-

directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science **239**:487–494.

31. **Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim.** 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science **230**:1350–1354.

32. **Sayler, G. S., M. S. Shields, A. Breen, E. T. Tedford, S. Hooper, K. M. Sirotkin, and J. W. Davis.** 1985. Application of DNA-DNA colony hybridization to the detection of catabolic genotypes in environmental samples. Appl. Environ. Microbiol. **49**: 1295–1303.

33. **Scharf, S. J., G. T. Horn, and H. A. Erlich.** 1986. Direct cloning

and sequence analysis of enzymatically amplified genomic sequences. Science **233**:1076–1078.

34. **Torsvik, V. L.** 1980. Isolation of bacterial DNA from soil. Soil Biol. Biochem. **12**:15–21.

35. **White, D. C.** 1986. Quantitative physiochemical characterization of bacterial habitats, p. 177–203. *In* J. S. Poindexter and E. R. Leadbetter (ed.), Bacteria in nature, vol. 2, methods and special applications in bacterial ecology. Plenum Press, New York.

36. **Yates, J. R., J. H. Lobos, and D. S. Holmes.** 1986. The use of genetic probes to detect microorganisms in biomining operations. J. Ind. Microbiol. **1**:129–135.