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Rapid method for the detection of genetically engineered microorganisms by polymerase chain reaction from soil and sediments

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A rapid and sensitive method for the detection of genetically engineered microorganisms in soil and sediments has been devised by *in vitro* amplification of the target DNAs by a polymerase chain reaction. A cloned catechol 2,3-dioxygenase gene located on the recombinant plasmid pOH101 was transferred to *Pseudomonas putida* MMB2442 by triparental crossing and used as a target organism. For the polymerase chain reaction from soil and sediment samples, the template DNA was released from a 100-mg soil sample. Bacterial seeded soil samples were washed with Tris-EDTA buffer (pH 8.0) and treated with a detergent lysis solution at 100°C. After addition of 1% polyvinylpolypyrrolidine solution, the samples were boiled for 5 min. Supernatant containing nucleic acid was purified with a PCR purification kit. The purified DNA was subjected to polymerase chain reaction, using two specific primers designed for the amplification of catechol 2,3-dioxygenase gene sequences. The detection limit was 10^2 cells per gram of soil. This method is rapid and obviates the need for lengthy DNA purification from soil samples.

Keywords: PCR; genetically engineered microorganisms (GEMs); *Pseudomonas putida*; catechol 2,3-dioxygenase; *xylE*

Introduction

Genetically engineered microorganisms (GEMs) have found widespread application in the synthesis of recombinant enzymes and other proteins, antibiotics, and industrially useful commodities and for remediation of chemically-polluted sites [10]. Widespread use of GEMs may lead to their inadvertent discharge in the ecosystem and hence it is important to develop methods to detect and monitor the fate of GEMs within a microbial community [6]. The potential risks to public health and the environment from a deliberate or accidental release to the environment has raised both scientific and public policy issues.

Traditionally, selective enrichment techniques have been used to enumerate specific bacteria [6]. However, this procedure does not guarantee efficient recovery of bacteria. Another widely used method is immunofluorescence, based on the use of specific antibodies to a GEM antigen [3]. The recent development of molecular-based detection techniques has greatly increased the ability to track microorganisms and introduced genetic material in natural environments. The insertion of marker genes, such as beta-galactosidase [2], allows tracking by cell extraction and subsequent growth on selective media. The use of marker genes requires cell extraction, growth, and expression of the gene, thereby countering many problems associated with traditional dilution plate enumeration techniques. In addition, DNA probes enable the detection of specific nucleotide sequences in the presence of high background levels of DNA. By using dot-blot hybridization, detection levels in the order of 5×10^4 cells g^{-1} of soil may be achieved [5]. DNA probes can also be used to detect specific microorganisms in soil, and when used in conjunction with polymerase chain reaction (PCR) methods, increases in the sensitivity of GEM detection by several orders of magnitude can be achieved [14]. DNA amplification via PCR, is widely used to enhance detection of genetically engineered bacteria in environmental samples [1,11,12,14,18,20]. Molecular biology methods, like dot-blot and colony hybridization, have been used to detect GEMs in environmental samples, eliminating the need for cell culture [1,11,13,14,18,20]. Although these methods are sensitive, there are limitations on the target gene sequence size and efficiency of DNA recovery [13,15,20].

Enzyme-dependent manipulation of nucleic acids, such as PCR, are hampered by the presence of inhibitory natural substances in environmental samples from which DNA is extracted, which may not be removed by standard DNA purification techniques [15–17,19]. Nonetheless, PCR has been employed to detect *Escherichia coli* [7,17], *Mycobacterium* [21], a *Frankia* sp [4], and a genetically engineered *Burkholdera cepacia* [14] introduced into soils and sediments.

In this paper we describe a rapid and sensitive method for the detection of GEMs by PCR from soil and sediments. The method can be used to release DNA from GEMs added to soil samples, without the interference of natural inhibitory substances found in these samples.

Materials and methods

Bacterial strains and growth conditions

Pseudomona putida MMB2442 was obtained from Dr Michel Bagdasarian (Michigan Biotechnology Institute,

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Lansing, MI, USA). The recombinant cosmid plasmid pOH101 (low copy number plasmid) encoding 2,3-catechol dioxygenase *xylE* previously named *bphC* [8] was transferred to *P. putida* MMB2442 by triparental crossing [8,9]. Organisms were grown overnight at 37°C in Luria-Bertani (LB) broth or on LB agar plates amended with appropriate antibiotics.

Soil characterization

The soils used in all experiments were collected from NCTR Campus, Jefferson, AR, USA. The nutrient and mineral contents of the soil were determined in the Soil Testing and Research Laboratory, University of Arkansas, Fayetteville, AR, USA. The soil was silt-loam and had a pH of 5.7. Nutrients in parts per million (wt/wt) were: calcium, 1491; potassium, 116; magnesium, 435; phosphorous, 14.5; sodium, 205; sulfate sulfur, 23.5; iron, 112; manganese, 8; copper, 5.7; zinc, 9.6; and nitrate, 33.5. The soil cation exchange capacity was 16 meq per 100 g, electrical conductivity 191 micromhos cm^{-1} and percentage base saturation 73.2. Bacterial numbers in the soil samples were determined by direct plating on LB agar plates.

Soil inoculation and PCR from soil samples

P. putida MMB2442(pOH101) was grown overnight at 37°C in LB broth, centrifuged, and resuspended in 10 mM sodium phosphate buffer (pH 7.0). The suspension was kept at room temperature for 2 h to deplete nutrient reserves. The cell suspension was then centrifuged again and the cell pellet was resuspended in deionized water. The suspension was serially diluted (10^6, 10^5, 10^4 and 10^3) in water before addition to soil or sediment samples. The cell concentration was verified by standard plate counts. Soil samples (100 mg) were inoculated with the serially diluted cells in 100 μl of deionized water. Control tubes contained either cells without soil (positive control) or soil with no added cells (negative control). Soil samples were washed twice with sterile TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), suspended in 400 μl of detergent lysis buffer (1% Tween 20, 5% Triton X-100, 10 mM Tris-HCl, 1 mM Na_2EDTA (pH 8.0)), and incubated in a boiling water bath for 10 min. The tubes were immediatley chilled in an ice-water bath for 10 min. The tubes were vortexed briefly and mixed with 200 μl of TESP (50 mM Tris-HCl, 20 mM disodium EDTA (pH 8.0), 100 mM NaCl, 1% (wt/vol) polyvinylpyrrolidone (Sigma Chemical Co, St Louis, MO, USA)) buffer, incubated for 5 min at 100°C, and cooled in ice for 5 min. The released DNA was separated by centrifugation at 10 000 × *g* for 5 min at 4°C to collect the supernatant. Then, 100 μl of TESP buffer was added to the pellet twice, mixed by brief vortexing, and the supernatant fluid was also collected after brief centrifugation. The DNA in the supernatant was purified by using a Qiaquick PCR purification kit (Qiagen, Inc, Chatsworth, CA, USA) according to the manufacturer's instructions. Purified DNA from the column was eluted with 50 μl of deionized sterile water. The DNA was concentrated to 10 μl using a Savant vacuum concentrator. Two microliters of concentrated DNA were used as a template for the PCR.

Primer selection

The recombinant strain *P. putida* MMB2442(pOH101) encoding 2,3-CDO (*xylE*) was selected for the amplification of the target gene because the plasmid was stable in the host for several generations without antibiotic and also has a low copy number. One pair of synthetic oligonucleotide primers CATF 5′-ATGAGAATAGGTCATGTTGC-3′ and CATR 5′-CGCCTGGTCTTCGGTCCAAGT-3′ targeting a 865-bp of 2,3-CDO (2,3-catechol dioxygenase, *xylE*) gene region, was used in the PCR. The primers were designed by computer analysis using sequences of the 2,3-CDO. The specificity of primers was confirmed by the GenBank database 'Blast' program. The primers were purchased from National Biosciences, Plymouth, MN, USA.

Amplification

The amplification reaction was performed by using a DNA thermal cycler (Perkin-Elmer model 480) and the GeneAmp kit with *Taq* DNA polymerase (Perkin-Elmer, Norwalk, CT, USA) in 0.5-ml micro-centrifuge tubes. The reaction mixture (50 μl total volume) consisted of 38.75 μl of sterile water, 5 μl of 10× PCR buffer (100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$, 0.1% (wt/vol) gelatin), 4 μl of deoxyribonucleoside triphosphates (2.5 mM each dATP, dTTP, dGTP and dCTP, 0.5 μl of each primer (stock concentration, 100 μM), 1–10 μl of template, and 0.25 μl (5 U μl^{-1}) of *Taq* DNA polymerase. After overlaying with sterile mineral oil, the samples were subjected to PCR amplification. Preincubation was at 95°C for 2 min. Thirty-five PCR cycles were run under the following conditions: denaturation at 94°C for 45 s, primer annealing at 65°C for 60 s, and DNA extension at 72°C for 90 s in each cycle. After the last cycle, the PCR tubes were incubated for 5 min at 72°C then at 4°C. Five microliters of the reaction mixture were analyzed by standard submarine gel electrophoresis (1.5% agarose; 5 V cm^{-1}), and the reaction products were visualized by staining with ethidium bromide (0.5 μg ml^{-1} in the running buffer). A reagent blank contained all components of the reaction mixture with the exception of template DNA, for which sterile distilled water was substituted. This step was included in every PCR procedure. The thermocycler, tips and pipetters used for preparing the PCR reagents and template DNA were kept in a different location from where the gels were loaded, stained and photographed. All reagents used in an experiment were taken from the freezer and discarded at the end of the day.

Isolation and identification of environmental bacteria

Bacterial populations in the soil samples were determined by serial dilutions that were plated on LB agar and incubated at 30°C for 48 h. Isolated colonies were identified by using Automicrobic System (bioMerieux Vitek, Hazelwood, MO, USA).

Restriction endonuclease digestions

Amplified samples (50 μl) were purified by using a Qiaquick PCR purification kit. Five microliters of the purified DNA samples were digested with *Eco*RI (Bethesda Research Laboratories, Gaithersburg, MD, USA) at 37°C for 1 h. The digested samples were analyzed by standard

submarine gel electrophoresis [9] on a 1.5% agarose gel at 5 V cm^{-1} for 2 h.

Results and discussion

The primary objective of this investigation was to develop a rapid and reliable method for tracking GEMs in soil by PCR. Therefore, our initial experiments focused on purification of template DNA from inoculated soil samples. Preliminary experiments to determine the optimum PCR amplification conditions utilized *P. putida* MMB2442 (pOH101) diluted in sterile water at different cell densities. The cells were boiled for 10 min in the presence of 0.1% Triton X-100 and cooled in an ice-water bath for 5 min and were used as a source of template DNA. Primer annealing temperatures close to the theoretical primer melting points allowed amplification of a single 865-bp product (Figure 1a, lane 2). In an initial analysis of the amplified *bphC* gene, the sequences were digested with restriction endonucleases known to cut the *P. putida* OU83 *bphC* gene. For successful digestion of the amplified product it was purified by using a Qiaquick PCR purification kit. The restriction digestion with *Eco*RI gave two bands of 591- and 274-bp (Figure 1a, lane 3). These results are in agreement with our nucleotide sequence (unpublished results) and restriction maps [9]. DNA extracted from an uninoculated soil sample was negative for the 865-bp PCR product.

Figure 1b shows the sensitivity and specificity of the PCR protocol using the CATF and CATR primers. The detection limit of the procedure, using these primers and amplification conditions, was 10 cells g^{-1} soil (Figure 1b, lane 9). Initially we tried to amplify the target gene from the inoculated soil samples by adding lysis solution and boiling for 10 min. We were unable to see amplification even at 10^8 cells. We used 100 mg of soil inoculated with different concentrations of GEMs. Figure 2a (lanes 2, 3 and 4) shows no amplification of target DNA at 10^6, 10^7, and 10^8 cells when we used the crude samples. Thermal cycling times, temperatures, $MgCl_2$, *Taq* polymerase concentration, primer concentrations, lysis method, and amount of soil samples were modified without success. It is also evident from Figure 2a that the soil itself was inhibitory for PCR.

Tsai and Olson [17] reported PCR-inhibitory substances in crude DNA preparations from environmental samples and their selective removal by column chromatography. Hilger and Myrold [4] developed a method to remove humic acids by electrophoretic separation of DNA and afterwards electroelution of DNA from the agarose gel. However, this method is time-consuming and less efficient. Polyvinylpolypyrrolidone (PVP) has also been used to remove the humic acids from DNA by adding it to agarose. This procedure for the removal of DNA from humic contaminants originally called for electroelution of DNA from the gel. We utilized a combination of detergent lysis, boiling, and the addition of a PVP solution. To optimize the lysis of microbial cells, a lytic protocol was developed that avoided the use of enzyme treatments (such as lysozyme, that may contain contaminant DNA) and sodium lauryl sulfate (SDS; that inhibits *Taq* polymerase activity [22]). The detergent lysis buffer contained Tris and EDTA to protect the DNA from nuclease activity produced by soil microorganisms. Low concentrations of Triton X-100 and

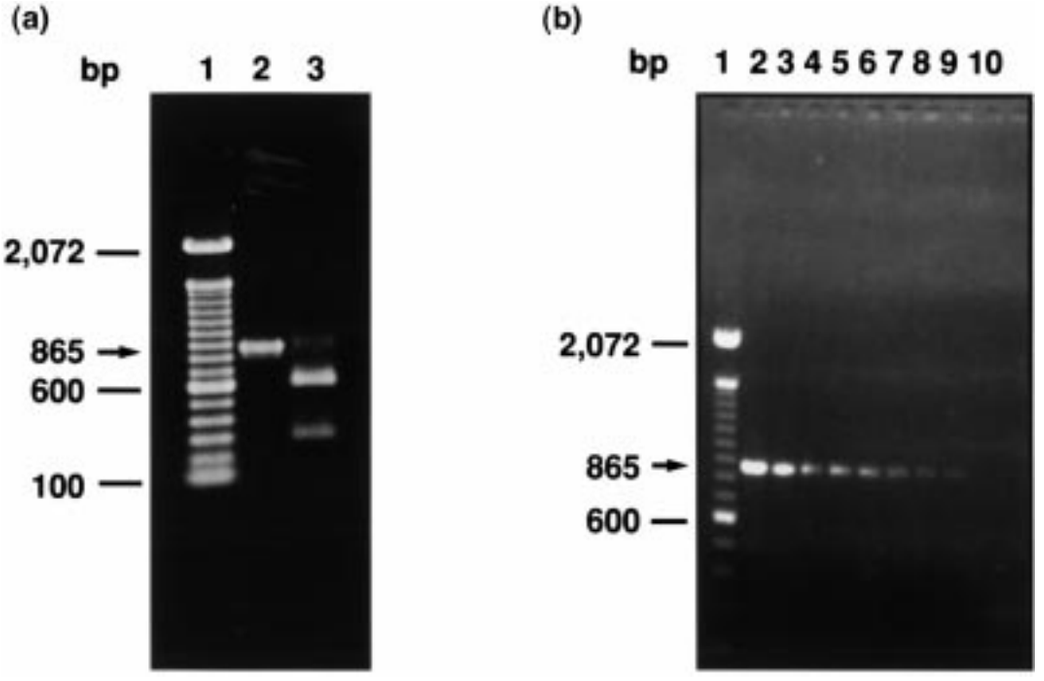

Figure 1 (a) Agarose gel electrophoresis of 865-bp amplified DNA from *P. putida* MMB2442 (pOH101) strain by primers CATF and CATR. Lane 1, 100-bp DNA ladder (BRL); lane 2, 865-bp amplified product from *P. putida* MMB2442 (pOH101); lane 3, PCR product digested with *Eco*RI (591- and 274-bp). (b) Sensitivity of the PCR protocol by amplifying the 865-bp region of 2,3-CDO gene by using CATF and CATR primers from *P. putida* MMB2442 (pOH101) strain. Lane 1, 100-bp DNA ladder (BRL); lane 2, 1×10^8 cells; lane 3, 1×10^7 cells; lane 4, 1×10^6 cells; lane 5, 1×10^5 cells; lane 6, 1×10^4 cells; lane 7, 1×10^3 cells; lane 8, 1×10^2 cells; lane 9, 10 cells; lane 10, no cells.

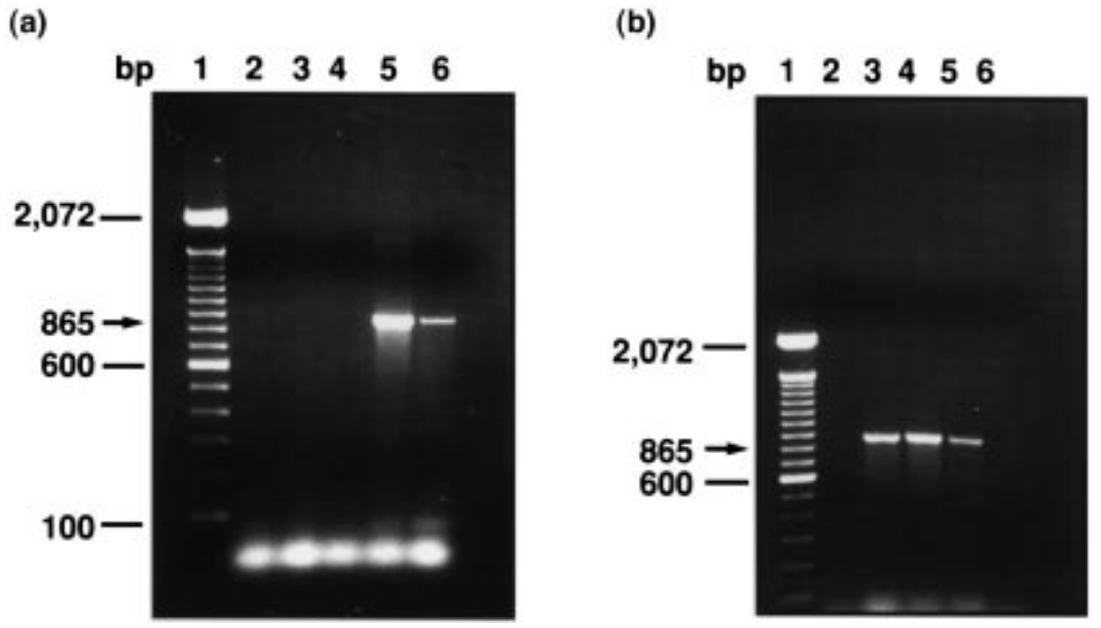

Figure 2 (a) Agarose gel electrophoresis of 865-bp amplified product from 100-mg seeded soil samples used directly in PCR cocktail without purification. Lane 1, 100-bp DNA ladder (BRL); lanes 2–4, 1×10^6 cells, 1×10^7 cells and 1×10^8 cells; lane 5 and 6, amplified from samples without soil 1×10^4 cells and 1×10^3 cells, respectively. (b) Agarose gel electrophoresis of 865-bp amplified product from template DNA purified from a 100-mg soil sample seeded with bacteria. Lane 1, 100-bp DNA ladder (BRL); lane 2, 1×10^1 cells; lane 3, 1×10^2 cells; lane 4, 1×10^4 cells; lane 5, 1×10^3 cells; lane 6, no cells (negative control).

Tween 20 have been used for the lysis of microorganisms by several investigators and both are mild detergents not inhibitory to *Taq* polymerase. NaCl helps disperse the solution, and insoluble PVP is used to remove humic acids and other phenolic impurities by adsorption. The soil sample was not subjected to a second boiling step, and the addition of the PVP solution failed to yield amplification of target DNA by PCR in our experiment. The treatment with TESP solution was found to be more effective for efficient DNA recovery and the PCR assay. Following the two-step lytic treatment, the extracted DNA contained a mixture of soil particles and cell debris. The first step consisted in sedimenting the lysate by centrifugation to eliminate the biggest particles; the DNA remained with the supernatant. However, to recover the DNA molecules which remained trapped in the debris, the pellet was washed twice with 100 μl of TESP buffer. This step is critical to the success of the procedure (data not shown).

DNA contamination of PCR solutions is a serious problem for PCR amplification, especially when small amounts of DNA are being amplified. The success of amplified product detection by PCR is also largely dependent on the degree of purity of the DNA solutions. The new method was devised to: (i) maximize DNA recovery; (ii) maintain the needed sensitivity while allowing the processing of many samples in a short period of time; and (iii) minimize contamination risks. Therefore, the extraction and purification steps were reduced to a minimum.

Purification and concentration can be generally achieved via selective adsorption of nucleic acids onto Qiagen PCR purification columns that contain silica gel-membranes. This purification step can be conducted in sterile conditions with DNA-free solutions, and high recovery efficiency. Eluted DNA can be concentrated to bring down the volume of DNA. However, the concentration step was not necessary at 10^6 cells. Figure 2b shows the PCR amplified product from various concentrations of cells. The detection limit was 10^2 cells g^{-1} (Figure 2b; lane 3). Soil samples having no *P. putida* (pOH101) were negative to PCR. The PCR primers for the target gene used were unable to amplify the endogenous bacterial population and also did not interfere with the assay. The predominant bacteria in soil samples were *Aeromonas sobria, A. hydrophila, Acinetobacter baumannii, Alcaligenes faecalis, Bacillus* sp, *Escherichia vulneris, Enterobacter sakazakii, Klebsiella pneumoniae, Pseudomonas putida, Rhodococcus* sp, and *Ochrobactrum anthropi*.

Successful PCR amplification of DNA from GEMs in soils and sediments requires a sequence of events, including cell lysis, removal of the DNA from soil or sediment, removal of humic acids and phenolic substances, prior to PCR amplification itself. Clearly, impairment of any step in this complex chain will diminish yields. This new method eliminates several lengthy steps and the detection limit was higher than with conventional plating procedures.

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