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Rapid and Sensitive Method for the Detection of *Mycobacterium chlorophenolicum* PCP-1 in Soil Based on 16S rRNA Gene-Targeted PCR

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A method based on 16S rRNA gene-targeted PCR and oligonucleotide probing was developed for detecting *Mycobacterium chlorophenolicum* PCP-1 in soil. The primers and probe were specific for PCP-1 in DNA extracts of three soils. The method allowed for PCP-1 detection in soil with a detection limit of 3×10^2 cells per g.

The pentachlorophenol degrader *Mycobacterium chlorophenolicum* PCP-1 (1, 2, 3) has been used for bioremediation of polluted soils (4, 5, 10). The detection of inoculant PCP-1 and assessment of its population dynamics, in conjunction with its pentachlorophenol-mineralizing activity, are important to understanding the efficacy of strain PCP-1 applications in soil. Since conventional detection techniques (i.e., plating) are not suitable for adequate enumeration of strain PCP-1 (5a), a molecular detection method was developed. This method is based on PCR amplification of a 243-bp region of the 16S rRNA gene and hybridization with an oligonucleotide probe.

Strain PCP-1 (DSM 43826) originated from pentachlorophenol-polluted sediment (2). Its taxonomy, physiology, and phylogeny have been described previously (1, 3). Cells were grown in DSM-65 medium (glucose [4 g], yeast extract [4 g], malt extract [4 g], demineralized water [1 liter]; pH 7.2) at 28°C on an orbital shaker (3 days), harvested by centrifugation (6,000 × *g*, 10 min, 20°C), and washed twice with sterile demineralized water. Cells were lysed in a bead beater (B. Braun, Melsungen, Germany). Glass beads with diameters of 0.17 to 0.18 and 0.09 to 0.11 mm and various shaking times (1.5, 3.0, and 4.5 min) were tested. Lysis was assessed by comparing the numbers of CFU on DSM-65 agar before and after treatment. In addition, it was monitored via immunofluorescence (9) with an anti-PCP-1 antiserum kindly provided by U. Karlson (Roskilde, Denmark). Following bead beating, strain PCP-1 DNA was extracted and purified according to standard procedures (11). For the design of PCR primers, variable regions of the 16S rRNA gene of strain PCP-1 were aligned with those of related organisms (3) with the EMBL database via the CAOS/CAMM Center (University of Nijmegen, Nijmegen, The Netherlands). The PCR mixture (50 μl) contained amplification (Stoffel) buffer (10 mM KCl, 10 mM Tris-HCl, pH 8.3), 3 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate, 0.35 μM each primer, 1 μl of target (either 10^5 to 10^6 boiled cells per ml or DNA), 0.5 to 1 U of *Taq* DNA polymerase, and Stoffel fragment (Perkin-Elmer Cetus, Norwalk, Conn.). The PCR was run for 35 cycles (1 min at 94°C, 2 min at 58°C, and 1 min at 72°C). Final extension was 7 min at 72°C. PCR was preceded by a hot start (7). PCR products were run on agarose gels (11) and subjected to Southern blotting onto nylon membranes (RPN Hybond 303N; Amersham, Buckinghamshire, United Kingdom). Blots were hybridized with the [γ-^{32}P]ATP end-labelled oligonucleotide probe or the [α-^{32}P]ATP randomly labelled (11) PCR product (positions 217 to 461) and washed at high stringency (for two 30-min rounds in 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 55°C).

Ede loamy sand (ELS) and Finnish peaty and sandy soils (from Kettula, Suomusjärvi, Finland), with 3.5, 14.7, and 0.7% organic matter (4, 14), respectively, were used. Strain PCP-1 cell suspensions were introduced at 3×10^2, 3×10^4, 3×10^5, 3×10^6, 3×10^7, 3×10^8, and 3×10^9 cells per g of ELS and at 3×10^6 cells per g in both peaty and sandy soils. Control soil portions received equivalent amounts of water. Soil moisture levels were set at pF 2, corresponding to 18, 62, and 9% moisture for ELS, peat, and sand, respectively. After 3 h as well as longer incubation times, soils were sampled to extract DNA by a direct method (12), with modifications of the glass beads and shaking times used and omission of the lysozyme and sodium dodecyl sulfate (SDS) treatments, as described below. Purification of the crude extracts was performed by steps of the protocol or via agarose gel electrophoresis followed by excision and further cleanup of the DNA band (15).

As lysis of PCP-1 cells is difficult, bead beating conditions were first optimized with pure cultures. With standard beads (diameter, 0.17 to 0.18 mm) bead beating at a 1:1 ratio (beads [grams] to volume of suspension [milliliters]) gave a low yield of disrupted cells (<70%), whereas a ratio of 2:1 enhanced cell disruption efficiency to around 94% after 4 min of treatment. The use of 0.09- to 0.11-mm-diameter beads at a ratio of 2:1 even increased strain PCP-1 cell lysis to >99% after 3 min of treatment. Cell density did not affect the efficiency of cell disruption. The use of SDS, either during or after the shaking procedure, also did not affect lysis. Treatment of ELS samples containing 3×10^9 PCP-1 cells per g by the improved procedure resulted in a lysis efficiency of >98%, as evidenced via dilution plating (made possible because of reduced indigenous bacterial growth). Also, about 10^6 PCP-1 cells detectable in soil via immunofluorescence showed >90% loss of cells by this procedure. Hence, the protocol for optimal lysis of *M. chlorophenolicum* PCP-1 cells in soil used bead beating with 0.09- to 0.11-mm-diameter glass beads for two 90-s rounds, omitting time-consuming lysozyme treatment as well as tedious SDS addition.

Alignment of the sequence of the 16S rRNA gene of PCP-1 with that of 16S rRNA genes of closely related bacteria indicated that variable parts of the region between nucleotides 213

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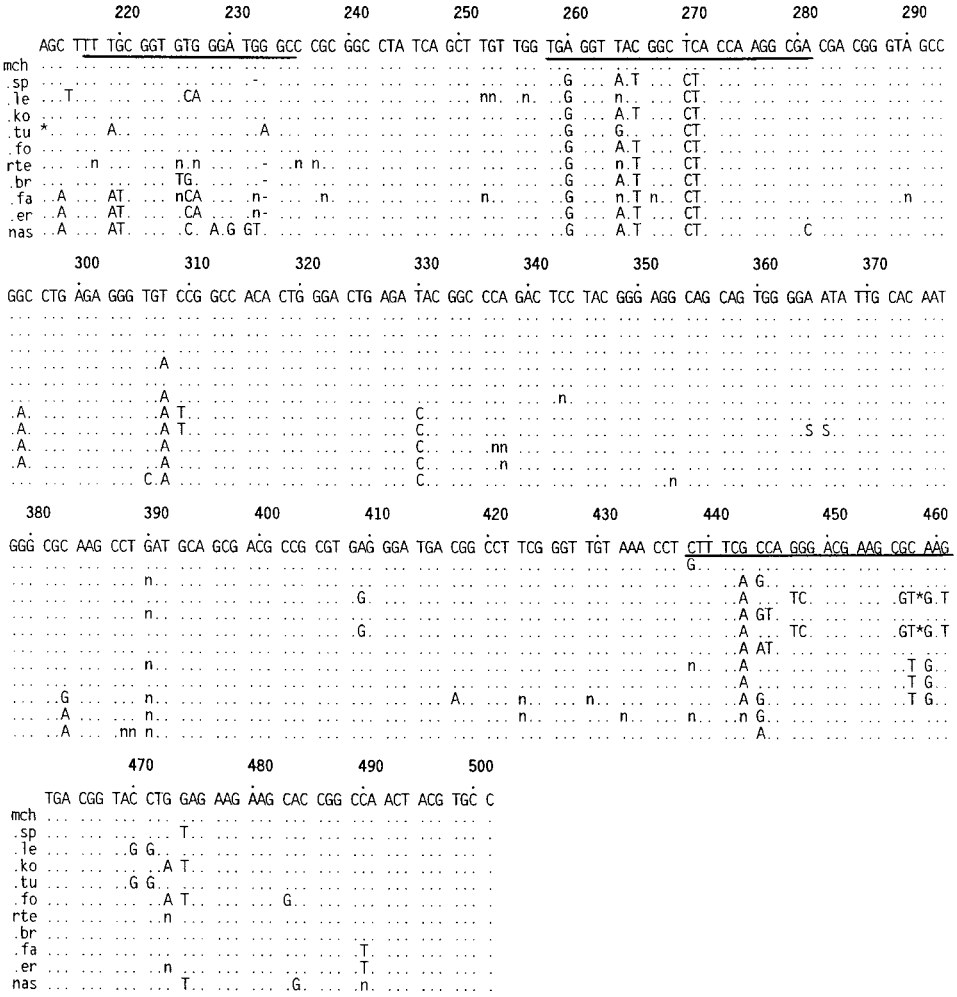

FIG. 1. Alignment of part of the 16S rRNA gene sequence of *M. chlorophenolicum* PCP-1 with corresponding regions of the phylogenetically closest species. This region contains the variable regions on which the three oligonucleotides (primers and a probe) used in this study were based. Abbreviations for species: mch, *M. chubuense*; .sp, *Mycobacterium sphagni*; .le, *Mycobacterium leprae*; .ko, *Mycobacterium komossense*; .tu, *Mycobacterium tuberculosis*; .fo, *Mycobacterium fortuitum*; rte, *R. terrae*; .br, *Rhodococcus bronchialis*; .fa, *Rhodococcus fascians*; .er, *R. erythropolis*; and nas, *N. asteroides*. The asterisk in the *M. leprae* sequence represents the following: 458-TGG Gnn TTC TCG-459. The asterisks in the *M. tuberculosis* sequence represent the following: 213-GC-214 and 458-CGG GTT CTC TCG-459. n, nucleotide.

and 472 (6) were suitable for the design of PCP-1-specific primers and a probe (Fig. 1). Three regions (i.e., 217 to 249, 256 to 297, and 435 to 472) were examined for specificity. Regions 256 to 297 and 435 to 472 were specific for strain PCP-1, since one to several mismatches were found with the closest relatives (except for homology to *Mycobacterium chubuense*, found in the former region). Region 217 to 249 was semispecific, since two strains with identical sequences were found. Two PCR primers, P217-f (positions 217 to 235; GAT GGA TCC TTT GCG GTG TGG GAT GGG C) and P460-r (positions 438 to 461; TTA AAG CTT CTT GCG CTT CGT CCC TGG CGA AAG), and an internal probe (positions 258 to 281; TGA GGT TAC GGC TCA CCA AGG CGA) were designed for the detection of PCP-1 in soil. Both primers contained restriction sites added to the 5′ ends. With this PCR system, an amplification product of 243-bp was generated on strain PCP-1 DNA, which hybridized to the internal oligonucleotide probe.

The P217-f and P460-r primers were tested for specificity by performing PCR on cell suspensions of 31 gram-positive bacteria including the following phylogenetically related as well as more distant taxons: *Arthrobacter globiformis* ATCC 8010 and NCIB 8602; *Bacillus cereus* FoTc-30; *Bacillus mycoides*; *Bacillus subtilis* 168 *trpC2*; *Clostridium beijerinckii*; *Corynebacterium bovis* ATCC 13722, ATCC 7715, and NCTC 3224; *Corynebacterium fascians* DSM 20131; *Corynebacterium flaccum*; *Corynebacterium michiganense*; *Corynebacterium* sp. strain C31; *Frankia* sp. strain ARI 3; *Frankia* sp. strain mu^{+}15; *Mycobacterium avium* DSM 43216; *M. chubuense*; *Mycobacterium flavum*; *Mycobacterium intracellulare* DSM 43223; *Mycobacterium marinum* DSM 43225; *Mycobacterium phlei* ATCC 354; *Nocardia asteroides* DSM 43005; *Nocardia* sp. strain ATCC 21197; *Rhodococcus equi*; *Rhodococcus erythropolis*; *Rhodococcus rhodochrous*; *Rhodococcus ruber*; *Rhodococcus rubropertinctus*; *Rhodococcus terrae*; *Rhodococcus percolatus* MBS1; and *Streptomyces lividans*. Agarose gel electrophoresis showed that the PCR primers did not give a 243-bp amplification product with 78% of the strains. However, a product was obtained with *Corynebacterium bovis* ATCC 7715, *M. intracellulare* DSM 43223, *M. marinum* DSM 43225, *N. asteroides* DSM 43005 and ATCC 21197, *R. erythropolis*, and *Corynebacterium fascians* DSM 20131. Hybridization with the oligonucleotide probe

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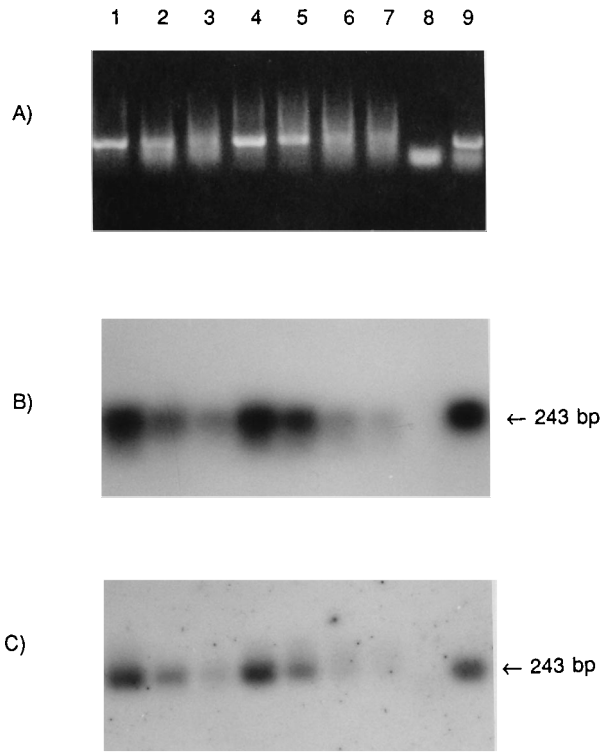

FIG. 2. Sensitivity of detection of *M. chlorophenolicum* PCP-1 in soil by PCR on DNA extracted from ELS inoculated with various densities of *M. chlorophenolicum* PCP-1 cells. (A) Agarose gel electrophoresis of the 243-bp PCR products amplified with the soil DNA extracts. Southern hybridizations of the gel shown in panel A with the 243-bp *M. chlorophenolicum* PCP-1 16S rRNA gene PCR product as a probe (B) and with the internal oligonucleotide probe (C) are also shown. Lanes contain PCR products generated with target DNA obtained from the following: ELS seeded with 3×10^9, 3×10^7, 3×10^5, 3×10^8, 3×10^6, 3×10^4, and 3×10^2 cells per g (lanes 1 to 7, respectively) and unseeded ELS (lane 8). Lane 9 contains *M. chlorophenolicum* pure DNA used as a target (positive control).

showed a signal only for the 243-bp product obtained with strain PCP-1 and not for products obtained with any of the other species. Therefore, the detection system composed of two PCR primers and an internal oligonucleotide probe was considered to be specific for strain PCP-1.

The PCR-oligonucleotide probe system applied to ELS and peaty and sandy soil DNA extracts allowed for the detection of strain PCP-1 in these soils inoculated with 3×10^6 cells per g. Amplification of DNA extracts of uninoculated soils did not result in PCR products, confirming the absence of cross-amplifiable background and hence specificity of the system for strain PCP-1 in soil. Recently, three other uninoculated soils, one of which had a history of pentachlorophenol contamination, also did not show 243-bp PCR products or hybridization signals with the probe.

Furthermore, the system detected as few as 300 PCP-1 cells per g of ELS (Fig. 2). Hybridization of the amplification products with either the 243-bp PCP-1-specific amplification product or the oligonucleotide probe produced signals which were consistent with one another (Fig. 2). In addition, signals of similar intensity were obtained in ELS inoculated with 10^6 PCP-1 cells per g after 2 and 14 days of incubation (results not shown). Since immunological detection indicated population size stability, these signals were most likely also indicative of stable strain PCP-1 populations in soil.

The modified lysis and DNA extraction method used here resulted in high efficiency levels of lysis of PCP-1 cells as well as recovery of DNA. The omission of detergents reduced the possible inhibition of *Taq* polymerase activity (16). Further, the DNA extracts obtained from soil were not severely sheared (average size, about 20 kb). The PCR-hybridization method allowed for the detection of PCP-1 cells in different soils, down to about 3×10^2 cells per g in ELS. This detection level indicates good sensitivity of the method, since it is on the order of the theoretical limit of detection. Using a similar approach, Tsai and Olson (13) were able to detect 5×10^2 *Escherichia coli* cells per g in soil. Picard et al. (8) improved the sensitivity of their PCR detection method for *Agrobacterium tumefaciens* (10^3 cells per g of soil), to 1 cell per g by using booster PCR. However, this protocol did not amplify a longer fragment (ca. 350 bp). The PCP-1 detection method developed in this study is rapid, sensitive, simple, and applicable to several soil types.

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