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Microscale Detection of Specific Bacterial DNA in Soil with a Magnetic Capture-Hybridization and PCR Amplification Assay

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A magnetic capture-hybridization PCR technique (MCH-PCR) was developed to eliminate the inhibitory effect of humic acids and other contaminants in PCRs targeting specific soil DNA. A single-stranded DNA probe, which was complementary to an internal part of the target gene, was used to coat magnetic beads. After hybridization in a suspension of soil DNA, magnetic extraction of the beads separated the hybrid DNA from all other soil DNA, humic acids, and other interfering soil components. The MCH was followed by PCR amplification of the specific target DNA. In barley rhizosphere soil, detection of a *lux* gene inserted in a *Pseudomonas fluorescens* strain could be demonstrated in nonsterile soil samples (0.5 mg). This corresponded to a detection of fewer than 40 bacterial cells per cm of barley root. The MCH-PCR technique greatly improves the current protocols for PCR detection of specific microorganisms or genes in soil because specific target DNA sequences from very small soil samples can be extracted and determined.

PCR (19) is a sensitive and specific method for direct detection of microorganisms or free DNA sequences in a variety of habitats such as food (25), natural water (2, 11), and soil or sediment (5, 18, 23). Much effort has been made to develop a PCR protocol suitable for soil samples, because this technique is most important in modern soil microbiology, e.g., for the detection of introduced microorganisms (23) or indigenous catabolic genes (5).

Since the pioneering work by Torsvik (26), isolation and detection of soil DNA have been based either on a direct lysis approach (21, 27) or on cell extraction prior to lysis (7, 8, 10). Modifications of the extraction protocol have been made to improve the quality (purity and length) of the DNA prior to further analysis (7) or to shorten the analysis time (10, 27). Nevertheless, PCR detection of specific DNA sequences has generally been based on native soil DNA, which has only partly been purified (5, 6, 17, 18, 27, 28). Such approaches have always encountered a strong interference by humic acids or other soil components, which are inhibitors of the *Taq* polymerase used in PCR amplification. To avoid this, laborious protocols including DNA purification (gradient centrifugation, column purification, and gel electrophoresis, etc.) or extensive sample dilution prior to PCR have been necessary in order to obtain an efficient and sensitive PCR (5, 6, 17, 18, 27, 28).

Microscale detection of specific microorganisms in heterogeneous environments such as bulk soil or rhizosphere soil surrounding plant roots is often desirable. With very small samples, typically 1 mg of soil, improvements of the existing DNA extraction protocols allowing PCR-based detection will be particularly important, since the target microorganisms or gene sequences are usually too few to produce a signal in standard DNA hybridization studies. Since PCR detection requires extensive purification of the target DNA (10, 17, 23, 28), the existing purification protocols would cause serious problems, e.g., incomplete recovery of DNA from the resin columns (27). With small amounts of target DNA, a relatively large fraction will be lost during the purification steps.

The purpose of this work was to develop a technique to separate specific target DNA from all other DNA, humic acids, and other interfering compounds in very small soil samples, thus facilitating detection of specific bacterial cells or genes by PCR. This novel method combines an initial DNA extraction purification step, including a solution hybridization with a single-stranded DNA probe on magnetic beads, and a subsequent PCR amplification step of the extracted target gene.

MATERIALS AND METHODS

Bacterial strains and PCR primers. *Pseudomonas fluorescens* DF57 (11D-4) and *P. fluorescens* DF57 (P2) were Tn*5-luxAB* transconjugants of *P. fluorescens* DF57 (22). *P. fluorescens* VKI171 (SJ132) was a Tn*5-luxAB* transconjugant of *P. fluorescens* VKI171 (9). The transconjugant strains were prepared as described by Kragelund et al. (12). In short, a promoterless transposon (Tn*5-luxAB* cassette from *Escherichia coli* DH5α pRL1063) was inserted into the chromosome of the recipient strain by triparental mating at 37°C, and transconjugants were selected on media containing appropriate antibiotics. The wild types DF57 and VKI171 were grown in pure LB medium (20), and the Tn*5-lux* mutants (9, 12) were grown in LB medium supplemented with 25 μg of kanamycin (Sigma Chemical Co., St. Louis, Mo.) per ml. All mutants showed the same growth characteristics in the API 20NE test system (BioMérieux SA, Marcy L'Etoile, France) and the same growth rates in LB medium as their wild types (9, 12).

Primers against the *luxA* gene from *Vibrio fischeri* (4) were selected to obtain uniform melting temperature values and GC contents (Table 1). One set of primers (*luxA6* and *luxA7*) were selected to amplify a product from nucleotide 237 to nucleotide 732; these primers are referred to as outer primers. Within this amplification product, a second set of primers (*luxA8* and *luxA9*) were selected to amplify a product from nucleotides 310 to 537; these primers are referred to as inner primers. The combined PCR amplification protocol consisting of a first cycle of amplification with *luxA6* and *luxA7* and a subsequent one with *luxA8* and *luxA9* represents a sensitive nested-PCR approach (15). The size of the PCR product with primers *luxA6* and *luxA7* is 495 bp. The subsequent PCR with primers *luxA8* and *luxA9* produces a product with a size of 227 bp.

Soil and rhizosphere systems. Two different soils with the following characteristics were used: (i) sandy loam (pH of soil water, 6.5; organic matter, 2.4%; clay, 10%; silt, 30.7%; fine sand, 31.9%; coarse sand, 24.1%) and (ii) loamy sand (pH of soil water, 7.0; organic matter, 1.1%; clay, 3%; silt, 10%; fine sand, 39.5%; coarse sand, 47.5%). The soil was passed through a 4-mm-pore-size sieve on the day of collection and stored in black plastic bags at 10 to 14°C until use. Barley seedlings (*Hordeum vulgare*) were grown in packed soil columns using 80 g (wet

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TABLE 1. Sequences and positions of oligonucleotides used in the MCH-PCR detection of *P. fluorescens* DF57 (11D4), *P. fluorescens* DF57 (P2), and *P. fluorescens* VKI171 (SJ132) (*luxA* gene of *V. fischeri*)

Oligonucleotide	Position in *luxA* gene	Sequence
luxA6	237–258	5′ ACA-GCA-CAC-CCA-GTT-CGA-CAG
luxA7	711–732	5′ CTC-CCG-ACA-AAC-ATC-TTG-CGC
luxA8	310–331	5′ GGA-ACC-GTT-CGA-GGG-CTA-TAC
luxA9	516–537	5′ TGT-CGT-ACT-TGC-GGA-CTC-AGC
luxA10[a]	364–463	5′ GAA-GAG-TCT-CGA-GCA-ATT-ACT-CAA-AAT-TTC-TAC-CAG-ATG-ATA-ATG-GAA-AGC-TTA-CAG-ACA-GGA-ACC-ATT-AGC-TCT-GAT-AGT-GAT-TAC-ATT-CAA-TTT-CCT-AAG

[a] Oligonucleotide with biotin molecule attached to the 5′ end with a spacer arm. The oligonucleotide is HPLC purified by the supplier.

weight, containing 20% water) of soil. The seeds were surface sterilized and immersed (30 min, room temperature) in a suspension of *lux*-carrying bacteria, which had been harvested from exponentially growing cultures and washed twice in phosphate-buffered saline (PBS)–0.9% NaCl. The cell concentration in the suspension was approximately 10^9 CFU/ml. The seeds were then grown in the soil for 7 days at 20°C, with 12-h light and dark cycles. In a different experiment, noninoculated seedlings were grown in natural soil to estimate the detection limit of the magnetic capture-hybridization (MCH)-PCR method. One-centimeter pieces of the roots including adhering soil were spiked with 5 μl of inocula of a dilution series of a *lux*-carrying *P. fluorescens* 11D-4. The root pieces were left on the bench for 30 min prior to further treatment.

Harvest of rhizosphere soil samples. Samples (1 cm) of root and loosely adhering rhizosphere soil were retrieved from the barley seedlings, suspended in 1 ml of PBS–0.9% NaCl buffer solution, and subjected to two successive sonications (10-s cycles) in a water bath (Metasohn 200; Struers, Copenhagen, Denmark). After the first cycle, the root fragments were transferred to new glass vials with buffer solution. The sonication was then repeated, and the root piece was transferred to new vials. All subsamples were collected separately. Aliquots (0.1 ml each) were spread plated on LB agar (20) with 25.0 μg of kanamycin, 20 μg of streptomycin, and 25 μg of nystatin (to inhibit fungal growth) per ml to enumerate culturable populations.

The remaining sample was either processed directly or frozen (−20°C) for later DNA extraction as described below.

DNA extraction from cultures and rhizosphere soil. DNA from pure culture was obtained by boiling one loopful of colony material formed overnight on LB agar plates as described by Brousseau et al. (3). The cells were boiled in 100 μl of sterile Milli-Q-purified water for 10 min and were centrifuged at 5°C for 10 min (20,000 × *g*). One hundred microliters of buffer solution with rhizosphere soil was also boiled for 10 min and then centrifuged at 5°C for 10 min (20,000 × *g*). Half of the supernatant (50 μl) was immediately removed and used for the MCH-PCR assay without further purification.

An optimal shearing of DNA prior to hybridization is important for optimal hybridization (1). Shearing of soil DNA to fragments with sizes of approximately 2,000 bp was performed by boiling in water. Shearing of DNA was compared by 1% agarose gel electrophoresis with lambda DNA that was either (i) boiled in Milli-Q-purified water, (ii) boiled in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), (iii) sonicated in Milli-Q-purified water, or (iv) sonicated in 6× SSC. Combinations of boiling and sonication did not further shear the DNA (data not shown).

Removal of humic acids in MCH-PCR. The efficiency of MCH upon removal of polymerase inhibitors was tested by the addition of commercial humic acids (Aldrich, Darmstadt, Germany) to DNA from *P. fluorescens* VKI171 (SJ132). Together with a control without humic acids, the samples were either amplified directly or magnetic capture hybridized, washed, and amplified as described below. Prior to PCR amplification, the DNA humic acid mixture was incubated overnight. The color of the dilution series with humic acids was compared with that of a series of extracted soil DNA which had been kept in a freezer (10, 16).

MCH-PCR: general outline. The general outline of the MCH-PCR procedure is given in Fig. 1. After conjugation of an internal probe to the magnetic bead (step 1), the coated beads are mixed with the nonpurified DNA solution to hybridize with target DNA sequences (step 2). By application of a magnetic field, the beads containing the target DNA are then extracted and removed from nontarget DNA and interfering compounds. During the first PCR annealing step (step 3), only one of the primers is applied. In the second and all subsequent PCR cycles (step 4), both primers are used.

MCH-PCR: preparation of internal probe and attachment of internal probe to magnetic beads. The internal probe (*luxA10* [Table 1]) spanning 102 bp of the *luxA* gene (4) was prepared by Oswel DNA Service (University of Edinburgh, Edinburgh, United Kingdom) with a biotin molecule on a five-carbon atom spacer arm incorporated on the 5′ end of the oligonucleotide. The oligonucleotide was purified by high-performance liquid chromatography (HPLC) by the supplier to ensure that all synthesized DNA actually carried the biotin molecule.

For a total of 20 reactions, 100 ng of the internal probe was attached to 400 μl of a 10-mg/ml suspension of magnetic M-280 streptavidin beads (Dynal, Skøyen, Norway). In all washing steps, a magnetic particle separator designed to fit Eppendorf tubes (Dynal MPC-E) was used. The beads were washed three times with 400 μl of 1× PBS–0.1% sodium dodecyl sulfate (SDS) (pH 7.3) (1× PBS is a 0.05 M phosphate buffer with 0.9% NaCl) to remove NaN_3 (preservative on the beads), which was followed by a single wash in 400 μl of Tris-EDTA (TE)–1 M NaCl (pH 8) and resuspension in 400 μl of TE–1 M NaCl. The internal DNA probe was then added, and the bead suspension was incubated for 60 min at room temperature in a hybridization oven. After incubation, the beads were washed three times with 400 μl of TE–1 M NaCl and resuspended in 400 μl of 0.125 M NaOH–0.1 M NaCl to ensure denaturation of the probe. The suspension was then incubated at room temperature for 15 min on the end-over-end mixer. The beads were finally washed three times with 400 μl of TE–1 M NaCl to remove traces of NaOH and were resuspended in 400 μl of water immediately before use.

MCH-PCR: hybridization and capture of target DNA. Samples with sheared target DNA (including positive control of cell material) and DNA-free samples (negative control for MCH-PCR) were hybridized for 4 h with 20 μl of the

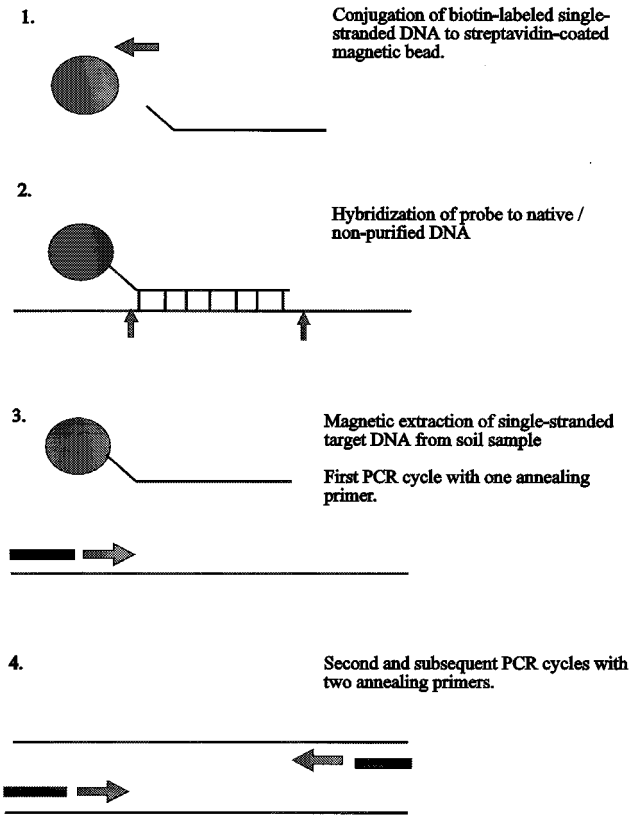

FIG. 1. MCH-PCR. 1. The biotin-labelled, single-stranded DNA is conjugated to a streptavidin-coated magnetic bead. 2. After production of the probe, a high-stringency hybridization to a nonpurified DNA sample is carried out in hybridization buffer. 3. After magnetic extraction, the recovered DNA hybrids are washed once and resuspended in purified water (in the first PCR cycle, only one primer is used). 4. In the second and all subsequent PCR cycles, both primers are used to amplify the desired product.

magnetic probe in a hybridization solution containing 5× SSC, blocking reagent (1% [wt/vol]) (Boehringer, Mannheim, Germany), *N*-laurylsarcosine (0.1% [wt/vol]) and SDS (0.02% [wt/vol]). Eppendorf tubes containing the samples were incubated in a rotating hybridization oven at 62°C.

After hybridization, the beads were concentrated by using an Eppendorf tube rack with a built-in magnet. The hybridization solution including soil particles and noncomplementary DNA was carefully withdrawn with a pipette. The beads containing probe and complementary DNA were resuspended in Milli-Q-purified water at room temperature and were again concentrated on the magnetic rack. After removal of the water with a pipette, the beads were resuspended in 50 μl of water and used directly for PCR analysis.

PCR amplifications of captured target DNA and detection of PCR product. The captured target DNAs from rhizosphere samples were PCR amplified by using the outer primer set (*luxA6* and *luxA7*). The PCR mixture contained 10 μl of sample with target DNA, 7.5 μl of PCR buffer without $MgCl_2$ (Perkin-Elmer Cetus, Birkerød, Denmark), 4 mM $MgCl_2$, 25 ng of each primer, 0.2 mM deoxynucleoside triphosphates (Boehringer Mannheim), and 1 U of Ampli*Taq* DNA polymerase Stoffel fragment (Perkin-Elmer Cetus). The reaction mixture was adjusted to a total volume of 75 μl with sterile water. In early experiments, Thermalase (KEBO, Albertslund, Denmark) was used with a $MgCl_2$ concentration of 2 mM as the only modification of the mixture described above.

The reaction mixture was overlaid with 2 drops of mineral oil (Perkin-Elmer Cetus), and samples were amplified in a DNA thermal cycler (Perkin-Elmer Cetus, model 480) with one cycle of 6 min of denaturation at 94°C, 45 s of annealing at 55°C, and 6 min of extension at 72°C. Another 39 cycles of 30 s of denaturation at 94°C, 45 s of annealing at 55°C and 1.5 min of extension at 72°C were performed. The final extension at 72°C was performed for 15 min. A 10-μl volume of the reaction product was separated by electrophoresis in a 3% NuSieve 3:1 gel (FMC BioProducts, Medinova, Denmark) in TAE buffer (20), stained in 0.5 μg of ethidium bromide per ml, and photographed. *lux* mix size marker was prepared from four individual PCR runs as described above with *P. fluorescens* VKI171 (SJ132) as the DNA target and combinations of the following primer sets: *luxA6* and *luxA7*; *luxA8* and *luxA9*; *luxA6* and *luxA9*; and *luxA8* and *luxA7*. The products of the PCR runs contained 495, 227, 300, and 422 bp, respectively. The products contained approximately the same amounts of DNA and were mixed at 1:1:1:1 to make up the *lux* mix size marker.

For Southern blot analysis, DNA was transferred from the gel to Hybond N hybridization membranes (Amersham, Birkerød, Denmark) by a capillary blotting technique (20); DNA probe for Southern blot analysis was labelled with ^{32}P (Amersham) on purified DNA from plasmid pRL1063 (12) containing the *luxAB* gene by using a random-primed kit (Boehringer Mannheim). The labelled probe was purified from free nucleotides by using the Spin Bind purification system (FMC Bioproducts). Prehybridization for 2 h and overnight hybridization were performed in hybridization buffer consisting of 5× SSC, 0.02% SDS, 0.1% (wt/vol) *N*-lauroylsarcosine, and blocking reagent (Boehringer Mannheim) at 68°C. The filters were washed twice for 5 min with 2× SSC–0.1% SDS at room temperature and twice for 15 min with preheated 0.1× SSC–0.1% SDS at 68°C. After this high stringency wash, the filters were exposed to X-ray film overnight.

Quantification of hybridization. The hybridization was quantified by using storage phosphor technology. The filters was exposed to the PhosphorImager screen (Molecular Dynamics, Albertslund, Denmark) for 16 h, and the stored energy was analyzed on PhosphorImager 425 as described by the supplier.

RESULTS

Removal of humic acids in MCH-PCR. By testing the efficiency of MCH on removal of polymerase inhibitors, it can be seen from Fig. 2B (lanes 2 to 7) that the direct PCR protocol was inhibited with a concentration of humic acids of approximately 64 ng per ml of reaction volume, while the PCR after MCH only was inhibited when the reaction mixture contained greater than 2 mg of humic acids per ml (Fig. 2A, lane 2). The MCH-PCR procedure thus worked well at much higher concentrations of humic acids corresponding to those of soil samples, since it was found that old DNA samples showed a brownish color corresponding to 16 to 80 μg of Aldrich's humic acid per ml. This result was obtained not only with Perkin-Elmer's Ampli*Taq* DNA polymerase but also with ICI's Thermalase (data not shown).

Detection of *lux*-marked *P. fluorescens* in barley rhizosphere by MCH-PCR. The indigenous microbial community in soil showed no hybridization to the *luxA* gene cassette, not even by the highly sensitive nested-PCR approach (15), and the absence of background signal allowed us to determine a detection limit for the MCH-PCR assay in rhizosphere soil. By spiking the *Pseudomonas* cell culture to harvested barley roots, the detection limit was 50 CFU present per sample (Fig. 3). Since only 1/20 of the extracting buffer solution was used in the MCH-PCR assay, the detection limit corresponds to 1,000 CFU on a 1-cm root piece. This result was obtained without using either Southern blotting or nested-PCR technology and is visible as a clear band in lane 6 in Fig. 3.

The two *Pseudomonas* mutant strains (11D4 and P2) colonized the seedlings equally well during 1 week in natural soil, resulting in 5×10^4 and 3×10^6 CFU/cm at the root base, which was measured after the first round of sonication. The corresponding numbers were 40 and 800 CFU/cm, at the root tip. After the second round of sonication, the CFU values were only 10% of those obtained after the first sonication.

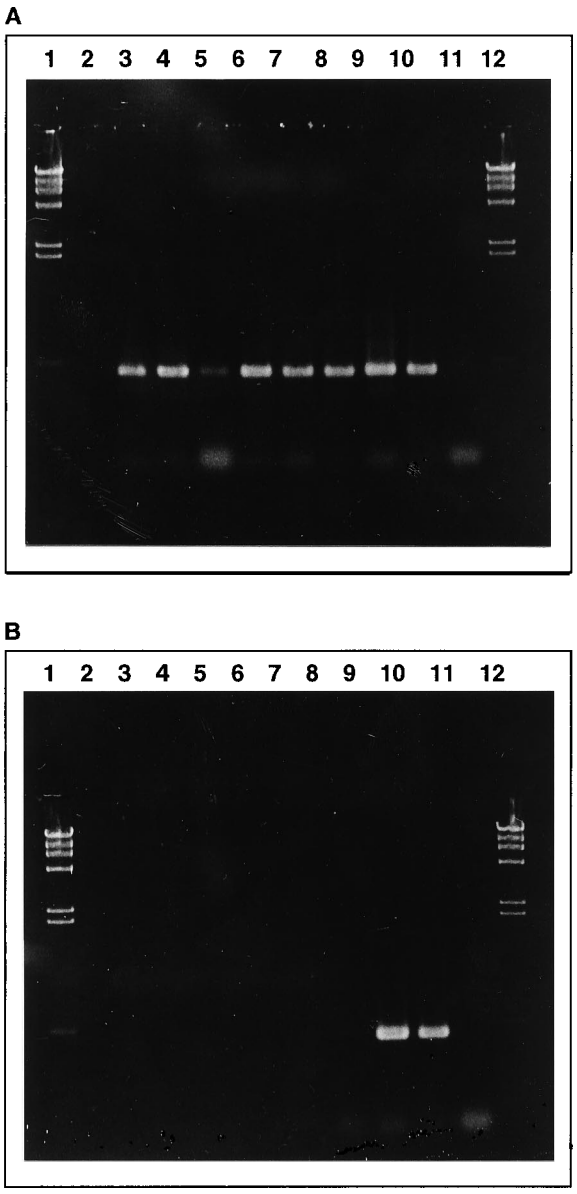

FIG. 2. Agarose gel electrophoresis of PCR amplification of DNA from *P. fluorescens* VKI171 (SJ132), which was amended with various amounts of humic acids. The samples were either PCR amplified directly (B) or MCH-PCR amplified (A). The lanes of both panels contain λ*Hin*dIII size marker (lanes 1 and 12), negative control (lane 11), positive control without humic acids (lane 10), and 10 mg, 2 mg, 400 μg, 80 μg, 16 μg, 320 ng, 64 ng, and 12 ng of humic acid per ml of reaction volume (lanes 2 to 9, respectively).

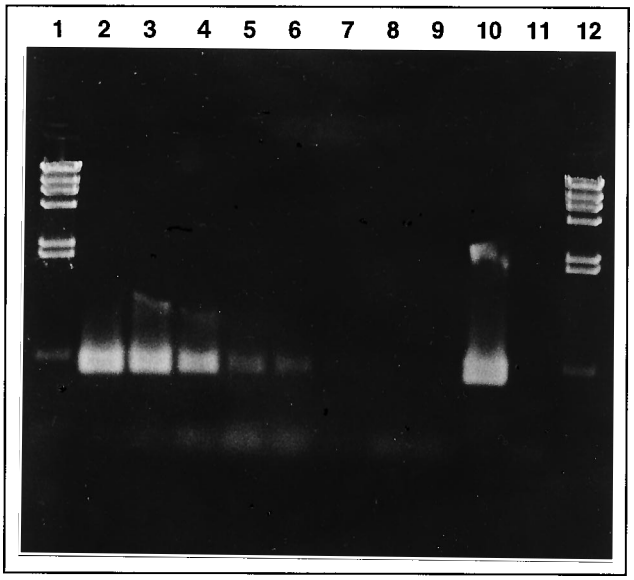

FIG. 3. Agarose gel electrophoresis of MCH-PCR amplification products of DNA from *P. fluorescens* DF57 (11D-4) in a barley rhizosphere. The strain was inoculated at different cell densities in 5 μl of buffer volume pipetted onto nonsterile barley roots. The cells were allowed to bind to the soil particles for 30 min before lysis and subsequent MCH-PCR amplification. Lanes: 1 and 12, λ-DNA-*Hin*dIII size marker; 2 to 8, 5×10^5, 5×10^4, 5×10^3, 500, 50, 5, and no cells added; 9, no rhizosphere sample added to magnetic probe (negative control in MCH-PCR); 10, MCH-PCR amplification on pure-culture DNA (positive control in MCH-PCR); 11, no DNA and no magnetic probe (negative control in PCR).

By the MCH-PCR method, the results from a first cycle of PCR that used agarose gel electrophoresis and Southern blotting are compared in Fig. 4A and B, respectively. It can be seen that the *luxA* genes are detected by both methods in samples from the root base (lanes 6, 7, and 8). In the samples from the root tip (lanes 3, 4, and 5), a visible amplification product was not obtained after the first PCR run as detected by ethidium bromide staining of the gel (Fig. 4A); however, a signal was clearly visible after Southern blot hybridization (Fig. 4B).

The quantification of Southern blot hybridizations correlated with the CFU values ($r = 0.87$), as shown in Fig. 5, when the data after both the first cycle and the second cycle of sonication were used. The detection limit by Southern blot technology is thus less than 40 CFU on a 1-cm root piece (two cells in the sample) obtained from 7-day-old seedlings.

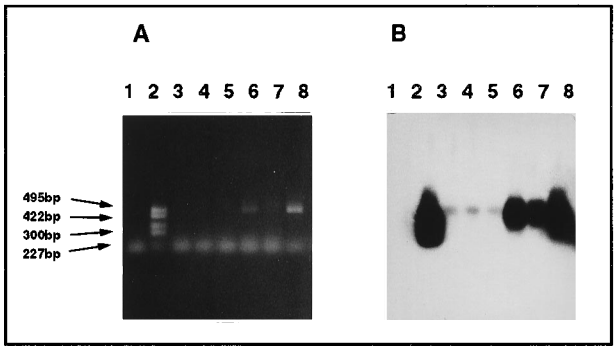

FIG. 4. (A) MCH-PCR detection of DNA from *P. fluorescens* DF57 (P2) in natural barley rhizosphere soil. The samples are from the first wash of the roots. Lanes: 1, negative control; 2, *lux* mix size marker (see text); 3 to 5, samples from root tip; 6 to 8, samples from root base. The corresponding counts calculated per centimeter of root were 90, 40, and 40 CFU (lanes 3 to 5, respectively) and 4.8×10^5, 5.3×10^4, and 3.3×10^6 CFU (lanes 6 to 8, respectively). (B) Southern blot of gel presented in panel A. The probes used were derived from *E. coli* DH5α (pRL1063), and the hybridization and washes were at high stringency as described in Materials and Methods.

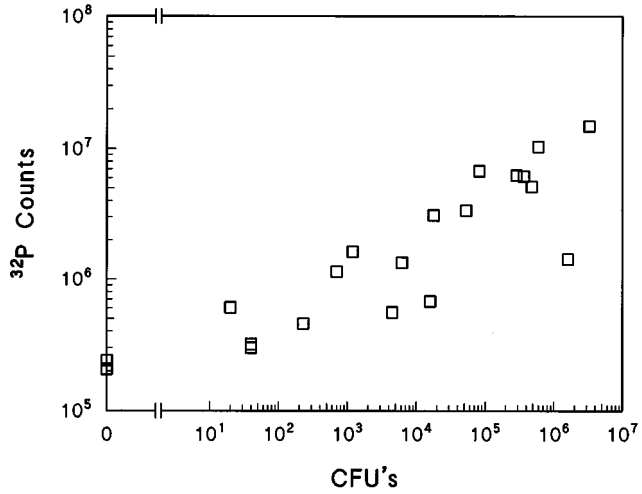

FIG. 5. ^{32}P counts from Southern blots plotted against the corresponding CFU counts in 20 samples, which were obtained after the first and second sonication cycles of natural roots colonized by *P. fluorescens* DF57 (P2) or *P. fluorescens* DF57 (11D4).

The nested-PCR approach that was used as a fast alternative to the Southern blot procedure (15) detected the presence of the *lux* gene in all of the soil samples described above (except the negative control) by using the inner primers *luxA8* and *luxA9*. However, the 227-bp-long DNA bands resulting from the nested-PCR amplification had similar intensities in all samples, regardless of the initial numbers of cells (data not shown). The detection level by nested PCR is thus possibly less than 40 CFU on a 1-cm root piece.

DISCUSSION

Advantages of the MCH-PCR technique. The strong need for a very simple detection method in soil environments based on DNA extraction and PCR amplification can be seen from the vast numbers of papers on this subject (17, 18, 23, 27). Most DNA extraction protocols attempt to obtain double-stranded high-molecular-weight DNA of high purity (7, 10, 23, 27). Such DNA is necessary in Southern blot experiments (7, 8), and the length of the DNA retrieved has been of prime concern in a recent evaluation of DNA extraction methods (13). Hence, some of the most efficient cell lysis procedures that use mechanical beating by beads or boiling may not be used to obtain large double-stranded DNA molecules. Beating techniques have thus been shown to shear the DNA (13), and the less harsh lysis of bacterial cells by lysozyme and hot detergent treatment is often chosen to avoid production of single-stranded DNA (7, 10). By comparison, the MCH-PCR procedure presented here is based on short, single-stranded DNA stretches which are easily and efficiently extracted from complex environments such as a soil or plant rhizosphere. Therefore, the MCH-PCR protocol takes advantage of a simple lysis step by boiling for 10 min. Such a procedure has been shown to give very reproducible and high-quality DNA samples from bacterial cultures for PCR-based DNA fingerprinting (3).

The presence of various compounds that inhibit the DNA polymerase in PCR amplification (5, 17, 21) must be consid-

ered in soil analysis, and humic acids are believed to strongly inhibit PCRs on soil DNA (17, 21, 24). The purity of the product by soil DNA extraction methods varies (13), and humic acids originating from different soils may have different inhibitory properties. By addition of commercial humic acid solution, Tebbe and Vahjen (24) found MICs at 0.64, 0.16, and 0.08 μg/ml for three *Taq* DNA polymerases from different suppliers (Boehringer Mannheim, Promega, and Perkin-Elmer, respectively). Using the same humic acid, we found that the Perkin-Elmer Ampli*Taq* DNA polymerase showed a MIC of 0.06 μg/ml, while ICI's Thermalase had a MIC of 8.33 μg/ml. Our data for inhibition of Ampli*Taq* polymerase from Perkin-Elmer are thus in the range of those found for the Stoffel fragment *Taq* polymerase from Perkin-Elmer as reported by Tebbe and Vahjen (24). When this large difference in the performances of the enzymes is considered, it should be noted that soil DNA purified in CsCl density gradients (10, 16) may contain approximately 80 μg of humic acid per ml but may still be of the highest purity (13). Only considerable dilution of such DNA (and the humic acid content) prior to direct PCR would give a reliable amplification product. This may be deduced from the effect of concentrating and diluting DNA samples obtained from environmental samples (2, 5).

Using the MCH-PCR protocol, we found no inhibition of the Ampli*Taq* DNA polymerase at humic acid concentrations of as high as 2 mg/ml. This concentration is more than 30,000 times higher than the concentration inhibiting a direct PCR amplification and more than 20 times higher than the approximate contents of undiluted soil DNA. It was previously shown that the hybridization reaction may be affected at high concentrations of humic acids (24); however, this effect was demonstrated after direct binding of 7 μg of humic acids to a hybridization membrane and therefore cannot be compared directly with the data from the MCH-PCR protocol. Since the MCH-PCR protocol selectively extracts the target DNA by magnetic force, it is likely that a number of other PCR inhibitors present in the soil also are removed.

The MCH-PCR protocol finally excludes the generation of false PCR amplification products, which may arise from mispriming in the presence of humic or phenolic compounds in the soil samples (23). With the high stringency hybridization during magnetic capture, the majority of nontarget DNA, which could have served as incorrect annealing sites for the primers, is removed.

Before eventual use of the MCH-PCR technique on samples other than rhizospheres, it should be considered whether parts of the technique should be mixed with parts of previously published DNA extraction protocols (5–7, 10, 21, 23, 27, 28). The use of 50 g of soil samples as in the procedure described by Jacobsen and Rasmussen (10) would, for example, most likely not work on a direct basis with MCH-PCR. However, in this case the MCH-PCR technique could be used on the crude DNA sample, with the CsCl density gradient purification step being omitted. A parallel approach can possibly be done with crude DNA from other protocols (5–7, 21, 23, 27, 28). Use of the MCH principle in combinations with reverse transcription-PCR to detect mRNA or virus is, moreover, an interesting future possibility.

Detection level by MCH-PCR in rhizosphere soil. On the basis of rhizosphere samples spiked with bacteria containing a *luxA* gene cassette, the detection of 1,000 CFU/cm of root was achieved with only one round of PCR, without Southern blot hybridization or nested PCR. The 1,000 CFU corresponds to 50 CFU in the PCR vial, since 1 cm of root was sonicated in 1 ml of buffer and only 50 μl was used for the MCH. No attempt was made to increase the volume of the hybridization reaction mixture and/or to lower the sample volume in the initial sonication step; however, this may have provided a lower detection limit. It has been reported that bacteria spiked to soil particles are more easily lysed than indigenous bacteria (27) and that PCR signals are more easily obtained from such a sample than from the indigenous soil population (5). When the applicability of the MCH-PCR method with regard to nonspiked soil samples was investigated, the *luxAB* gene sequence was still useful as a target, since it is absent in natural soil. The model system with barley seedlings growing for 1 week after seed inoculation of the *luxAB*-marked bacteria was, therefore, an appropriate control of the detection limit.

The high level of sensitivity of detection by the MCH-PCR protocol was clearly verified in the experiment with *luxAB*-labelled *Pseudomonas* cells colonizing the barley roots. Hence, only a few CFU obtained per centimeter of root tip correspond to a clear signal after Southern blot hybridization of the MCH-PCR products (Fig. 4, lanes 3 to 5). On the basis of plotting of ^{32}P-labelled quantification against CFU (Fig. 5), it can be seen that a MCH-PCR signal arose from several samples containing between 0 and 1,000 CFU/cm of root. The detection limit by the MCH-PCR technique on natural samples is consequently very satisfactory and even better than that obtained with the spiked rhizosphere samples. The MCH-PCR detection of specific bacteria or DNA reported in this paper is still only semiquantitative. A recent paper by Leser et al. (14) has demonstrated that it is possible to make quantitative DNA detection by PCR in aquatic samples with an internal DNA standard. A quantitative MCH-PCR is probably possible, since no DNA can be lost in the purification steps taking place in a single reaction tube. The MCH-PCR method was developed to solve the difficult task of analyzing specific DNA in small natural soil samples, and the MCH-PCR protocol clearly has a unique sensitivity and is suitable for use in very small samples of soil (approximately 1 mg).

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