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# Estimation of the Abundance of an Uncultured Soil Bacterial Strain by a Competitive Quantitative PCR Method

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**Strain EA25 was identified in a clone library of bacterial 16S rRNA gene sequences that had been amplified from DNA extracted from soil collected in eastern Washington State. EA25 was subsequently shown to be related to members of the genera *Planctomyces* and *Chlamydia* and most closely related (93% similarity) to strain MC18, a strain identified in an Australian soil sample (W. Liesack and E. Stackebrandt, J. Bacteriol. 174:5072–5078, 1992). A competitive quantitative PCR method developed by Zachar et al. (V. Zachar, R. A. Thomas, and A. S. Goustin, Nucleic Acids Res. 21:2017–2018, 1993) was used to estimate the abundance of this uncultured strain in soil. An estimation of the abundance of EA25 was based on the number of copies of the sequence in the DNA extracted and the efficiency of the DNA extraction. In addition, amplification rates of *Escherichia coli* DNAs added to soil were shown to be similar to those of DNAs from laboratory cultures of *E. coli*. The number of EA25 16S rRNA genes was estimated to be $2.17 \times 10^8$ copies per g of soil, suggesting that strains similar to EA25 and the similar Australian strain could be widely distributed and present in significant numbers in soils from temperate regions. This represents the first enumeration of 16S rDNA copies from an uncultured strain in soil.**

The cloning and sequence analysis of 16S rRNA genes (rDNA) directly isolated from environmental samples have proven to be powerful tools in detecting previously undescribed and potentially unculturable bacterial strains (1, 2, 7, 11, 21, 29, 34). The most extensive study to date employing 16S rDNA sequencing to describe the community structure of a soil was conducted with an Australian soil sample, in which Liesack and Stackebrandt (17) identified numerous previously undescribed bacterial rDNA sequences. Among these sequences were those belonging to members of the family *Planctomycetaceae*, previously thought to be present only in aquatic environments, and to a novel group that has a common ancestor with the genera *Planctomyces* and *Chlamydia*. The observation that these groups were found in a clone library constructed from soil DNA raises questions regarding the distribution of these groups in soils, their numbers, and their roles in soil communities.

The investigation of the numbers and distribution of novel bacterial groups, such as those described by Liesack and Stackebrandt (17), will shed light on the relative importance of these groups in soils and are necessary preliminaries to studies determining the roles of these groups within soil microbial communities. If these groups are widely distributed on a global basis and are present in significant numbers, they are likely to be important and integral members of many soil communities.

Since significant portions of many soil communities have never been cultured (32), the detection and enumeration of many of these groups are limited to the detection and enumeration of their rDNAs. Information regarding the concentrations of uncultured groups may be obtained by hybridization of group-specific oligonucleotide probes to nucleic acids directly isolated from the environment (12, 23), but this approach is not very sensitive and would not be likely to detect species in low abundances (1). Alternatively, group- or species-specific PCR primers could be designed and used in any of a number of quantitative PCR (QPCR) approaches to detect and enumerate relatively low concentrations of uncultured microorganisms.

A number of approaches to QPCR have been developed over the last several years, and two general QPCR approaches have been applied to microbial ecology: (i) those that are based on most-probable-number analysis (22, 30), in which PCR is used to screen for the presence of the target sequences in samples diluted to extinction with several replicates of each dilution, and (ii) those that use a competitive QPCR approach (8, 16), in which an internal standard (competitor) with a known DNA sequence and mass is added to the sample prior to PCR. In competitive QPCR, the competitor and the target are amplified together and the ratio of the final mass of the competitor to its initial mass is compared with the final mass of the target sequence so that the starting mass of the target DNA can be approximated. Ideally, the competitor should have a size and primer binding sites similar to those of the target so that the two DNAs will compete for reagents, such as enzymes, primers, Mg, and deoxynucleoside triphosphate (dNTP).

The first approach, most-probable-number PCR, is a relatively simple procedure that does not require the construction of a competitor, but it is rather cumbersome because of the number of replicates and dilutions that are required. The large number of dilutions and replicates required makes the rapid analysis of multiple samples difficult, and most-probable-number analysis is by nature relatively imprecise (5). Competitive QPCR has the advantage that, once the competitor has been constructed, multiple samples can be analyzed relatively easily and with a higher degree of precision than would be allowed by most-probable-number analyses.

We have been interested in using approaches similar to those employed by Liesack and Stackebrandt (17) to evaluate the response of soil microbial communities to exposure to

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organic contaminants. As part of this study, microbial DNA from an uncultivated field soil in eastern Washington State was directly isolated and a clone library containing 16S rDNA sequences was constructed and partially sequenced. Several members of this clone library showed high-level similarities with the novel groups found in the Australian soil sample, and one of these was chosen for enumeration by a competitive QPCR method as a preliminary to detailed ecological studies on this strain. To validate the method, we compared the relative rates of amplification of DNA isolated from soil with those of DNA from a pure culture and have applied two independent methods to evaluate the efficiency of purification of soil DNA. To the best of our knowledge, this represents the first application of competitive QPCR to enumerate an uncultured, indigenous strain in soil.

## MATERIALS AND METHODS

**Soil collection and isolation of soil DNA.** Surface soil was collected in August 1994 from an uncultivated pasture approximately 15 miles (24 km) north of Pullman, Wash. The soil, characterized as Palouse silt loam soil, was mixed, sieved (2-mm nominal pore size), and stored in a polyethylene bag at −20°C. The characteristics of this soil have been described elsewhere (35).

Total soil DNA was extracted and purified by a modification of the procedure described by Xia et al. (35) and described below. One gram of soil sample was washed twice with washing solution (0.12 M sodium phosphate buffer, pH 8.0) to remove extracellular DNA and to disperse the soil. Eight milliliters of lysis solution I (0.15 M NaCl, 0.1 M EDTA [pH 8.0], 10 mg of lysozyme per ml) was added and incubated at 37°C with occasional mixing for 1 to 2 h, and then 8 ml of lysis solution II (0.1 M NaCl, 0.5 M Tris-HCl [pH 8.0], 10% sodium dodecyl sulfate) was added. The sample was frozen at −70°C and thawed in a 65°C water bath, and this freezing and thawing cycle was repeated three times (33). The lysate was centrifuged at 12,000 × *g* for 10 min with a Beckman (Fullerton, Calif.) J2-21 centrifuge, and this was followed by filtering through a single sterile Kimwipe into a fresh tube.

The lysate was brought to a final concentration of 1% CTAB (hexadecyltrimethyl ammonium bromide; Sigma Biochemical, St. Louis, Mo.) and 0.7% NaCl (i.e., 2.7 ml of 5 M NaCl and 2.1 ml of 10% CTAB in 0.7 M NaCl were added to 16 ml of lysate). The lysate was mixed and incubated at 65°C for 10 min, and this was followed by extraction with an equal volume of $CHCl_3$-isoamylalcohol (24:1). An equal volume of 13% polyethylene glycol (molecular weight, 8,000) in 1.6 M NaCl was added to the upper phase and held on ice for 2 h. The sample was then centrifuged at 12,000 × *g* for 15 min, and the pellet washed once with 70% ethanol. The pellet was briefly dried at room temperature and dissolved in 3 ml of TE (10 mM Tris-HCl, 1 mM EDTA [pH 8.0]). A 440-μl volume of 10 M ammonium acetate was added for a final concentration of 1.5 M ammonium acetate, and the sample was incubated at room temperature overnight. The mixture was centrifuged in a microcentrifuge at full speed for 10 min, and the pellet was discarded. Two volumes of ethanol were added to the supernatant. The mixture was kept at −20°C for 2 h, and the precipitated DNA was recovered by centrifugation. The pellet was washed with 70% ethanol and dried at room temperature.

The dried pellet was dissolved in 60 μl of TE and electrophoresed in 0.8% SeaPlaque agarose (FMC Bioproducts, Rockland, Maine) containing 0.2% polyvinylpyrrolidone (Sigma) in a modification of the procedure described by Young et al. (36). High-molecular-weight DNA (greater than 20 kb) was excised and placed in preweighed 1.5-ml microcentrifuge tubes. The same mass of water was added, and the sample was heated at 65°C for 10 min and used for amplification by PCR.

**Amplification and cloning of 16S rDNA.** The 16S rRNA genes were amplified by PCR with universal bacterial 16S rDNA primers with the following base compositions (4, 14): 5′-CUACUACUACUATNANACATGCAAGTCGAKCG-3′ (68FU) and 5′-UACUACUACUACACGGCGGTGTGTRC-3′ (1406RU). The 12 nucleotides at the 5′ end of each primer serve as adapter for uracil DNA glycosylase (GIBCO BRL, Grand Island, N.Y.) cloning. PCR was performed on a DNA thermal cycler (model 480; Perkin-Elmer, Norwalk, Conn.). PCR amplification was conducted in a total volume of 20 μl containing 0.5 pmol of each primer per μl, 200 μM (each) dNTP, 1.5 mM MgCl, 1× *Taq* buffer and 1 U of *Taq* polymerase (Promega, Madison, Wis.). The DNA templates were first subjected to a denaturation step for 4 min at 94°C. The subsequent 35 cycles consisted of a 1-min denaturation step at 94°C a 1-min annealing step at 55°C, and a 2-min primer extension step at 72°C. Negative controls with SeaPlaque instead of DNA showed no amplification.

PCR products were run on a low-melting-point agarose gel and viewed with ethidium bromide, and bands in the proper size range (ca. 1,338 bases) were excised, purified with Geneclean II (Bio101, Vista, Calif.), digested with uracil DNA glycosylase to remove uracil, and cloned into the pAMP vector of a CLONEAMP kit according to the manufacturer's instructions (GIBCO BRL). Clones containing appropriate-sized inserts were identified by agarose gel electrophoresis of PCR products obtained from host lysates by PCR with primers complementary to the vector at sites flanking the insertion site. Unique clones were identified by restriction fragment length polymorphism analysis of the inserts (9). Seven clones representing the dominant restriction fragment groups were selected, and 1,300 nucleotides of each of these clones were sequenced by PCR cycle sequencing of quick-prepped recombinant plasmids. Cycle sequencing was performed with either the *fmol* cycle sequencing kit (Promega) or the GIBCO BRL cycle sequencing kit according to the manufacturer's instructions. Both strands of the 16S rDNA of EA25 were sequenced twice by the Molecular Genetics Instrumentation Facility of the University of Georgia, Athens, using internal sequencing primers suggested by Lane (14).

The sequences obtained were manually aligned with those in a database of previously determined rDNA sequences obtained from GenBank (3) and the University of Illinois ribosomal RNA database project (20).

Sequences were submitted to the CHECK-CHIMERA program of the University of Illinois ribosomal RNA database project to detect the presence of possible chimeric artifacts. These clones were placed into appropriate phylogenetic relationships by a neighbor-joining method using the top-scoring sequences from searches of the two databases listed above.

**Postlysis extraction DNA efficiency.** The efficiency of extraction of DNA by our isolation procedure was evaluated in two ways: (i) by addition of known amounts of *Escherichia coli* genomic DNA prior to lysis and subsequent quantitation of recovery of the added DNA by QPCR (described below under QPCR) and (ii) by determining the recovery of known amounts of added labeled lambda bacteriophage DNA.

For evaluation of recovery efficiency by the addition of labeled lambda DNA, lambda bacteriophage DNA (48.502 kb) was digested with *Xba*I (Boehringer, Mannheim, Germany), yielding two fragments of approximately 24.5 and 24 kb. These two fragments were labeled with $^{32}P$ by the action of terminal deoxynucleotidyl transferase (GIBCO BRL) and [α-$^{32}P$]dCTP according to the vendor's instructions. The labeled DNA was partially purified by precipitation in a one-fifth volume of 10 M ammonium acetate and 2 volumes of ethanol. The labeled DNA was recovered by centrifugation, washed with 70% ethanol, dried under vacuum at room temperature, and then resuspended in $H_2O$.

The labeled DNA was purified of unincorporated nucleotides by repeated centrifugation in Centricon-100 tubes (Amicon, Beverly, Mass.) (19). Centrifugation in Centricon-100 tubes was repeated until the counts per minute of the filtrate represented less than 1% of the total counts per minute. The labeled DNA was added to three replicates of the soil-lysis solution I slurry immediately prior to lysis at a rate of approximately $10^4$ cpm/g of soil. Following extraction and purification of the soil DNA according to our method, the amount of the radioactivity recovered in individual agarose bands was determined by scintillation counting.

**QPCR.** Primers specific to the 16S rDNA of *E. coli* (EC30F and EC1044R) and of EA25 (EA25FB and EA25-1150R), a clone identified from analysis of sequences amplified from soil, were designed with the following sequences: primer EC30F, 5′-GCTTGCTTCTTTGCTGAC-3′; primer EC1044R, 5′-TGGCAAACCAGGATAAGC-3′; primer EA25FB, 5′-GCAATACAGGGAAAAGTCTAGC-3′; and primer EA25-1150R, 5′-AGTGCTCGAGCCTCACGGCTC-3′. The specificities of these primers were checked by amplification of soil DNA. No products were observed with the *E. coli* primers, and sequencing of the amplification product obtained with the EA25 primers confirmed that only EA25 sequences were amplified.

QPCR was conducted by the method developed by Zachar et al. (38). For QPCR of EA25 sequences, competitor DNAs for *E. coli* and EA25 were constructed as follows. The 16S rDNAs of *E. coli* and EA25 were amplified with *E. coli*-specific 16S rDNA primers (EC30F and EC1044R) and EA25-specific 16S rDNA primers (EA25FB and EA25-1150R). EA25 primer specificity was checked by cloning the amplification product from soil as described above, and approximately 600 bp of one clone was sequenced. The amplification product was 100% similar to EA25 over the sequenced range. The PCR-generated 16S rDNA amplification products were cloned into the TA cloning vector pCRII (Invitrogen, San Diego, Calif.) and were designated pEC for the *E. coli* rDNA clone and pEA25 for the EA25 rDNA clones. A 121-bp fragment isolated from pBR322 by digestion with *Eco*RII was inserted into a *Sac*II restriction endonuclease site within the 16S rDNA sequence, yielding the pEC-C competitor for *E. coli* and pEA25-C competitor for pEA25. The EA25 competitor, pEA25-C, was 150 bp smaller than the original clone, indicating that a second *Sac*II restriction endonuclease site was present in the 16S rDNA insert. The additional *Sac*II site resulted in the loss from the clone of approximately 270 bp internal to the EA25 16S rDNA gene. The resulting inserts for pEA25 and pEA25-C were 1,125 and 855 bp, respectively.

A single set of standard samples containing a known amount of target DNA was prepared. Each reaction mixture was spiked with a constant amount of competitor, and all samples were amplified in triplicate PCRs. The 16S rDNA sequences were amplified by PCR under the conditions described above under Amplification and cloning of 16S rDNA, with the inclusion of acetamide to 5% (wt/vol) (24). PCR amplifications were conducted in the presence of 10 μCi of [α-$^{32}P$]dCTP so that the radiolabeled products could be quantitated by scintillation counting following separation by agarose gel electrophoresis (see below). The DNA templates were first subjected to a denaturation step for 4 min at 94°C.

The thermal cycling conditions were as follows: denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min. For EA25-specific primers, the annealing temperature used was 60°C. Unless specified otherwise, 30 cycles of amplification were carried out for QPCR.

Not all of the amplification products obtained by these reactions could be visualized by ethidium bromide staining, making subsequent excision of the bands for scintillation counting difficult. In order to easily visualize the locations of the appropriate bands on the gel, nonlabeled amplification products obtained from separate reactions were added to the experimental samples following amplification and prior to electrophoresis. This allowed the amplification products to be visualized by ethidium bromide staining, allowing for a more precise identification of the location of the appropriate band for excision. This additional DNA was used only as a marker for locating the bands and did not interfere with quantitation of the experimental bands by scintillation counting. PCR products were separated by electrophoresis through a 1% agarose gel containing ethidium bromide (1 μg/μl) in Tris-borate-EDTA buffer. The bands of target DNA and competitor in the same lane (as identified by the added nonradioactive amplification products) were excised and dissolved in 10 ml of scintillation cocktail (Ready Gel; Beckman) overnight. The amount of radioactivity in each band was analyzed with a liquid scintillation counter (Packard model 1900 CA).

In order to construct a calibration curve for each amplification, the logarithm of the ratio of the radioactivity (as counts per minute) of DNA in the target DNA band to that in the competitor band was plotted against the logarithm of the mass of the input target DNA (38). This calibration curve was calculated by a least-squares analysis to determine the amount of target DNA in the unknown sample by using the ratio of target DNA to competitor PCR product. Since a known concentration of pure EA25 DNA is not available, the calibration curve for this sequence used pEA25, the cloned 16S rDNA sequence of EA25.

The amplification rates of *E. coli* DNA in the purified soil DNA extract and those of *E. coli* genomic DNA and pEA25 isolated from pure cultures (as described above) over a range of PCR cycles using the primers and cycling conditions described above were determined. The amplification rates of EA25 DNA in the purified soil DNA extract were determined similarly.

**Nucleotide sequence accession number.** The nucleotide sequence data for strain EA25 have been submitted to the GenBank and EMBL nucleotide sequence databases under accession number U51864.

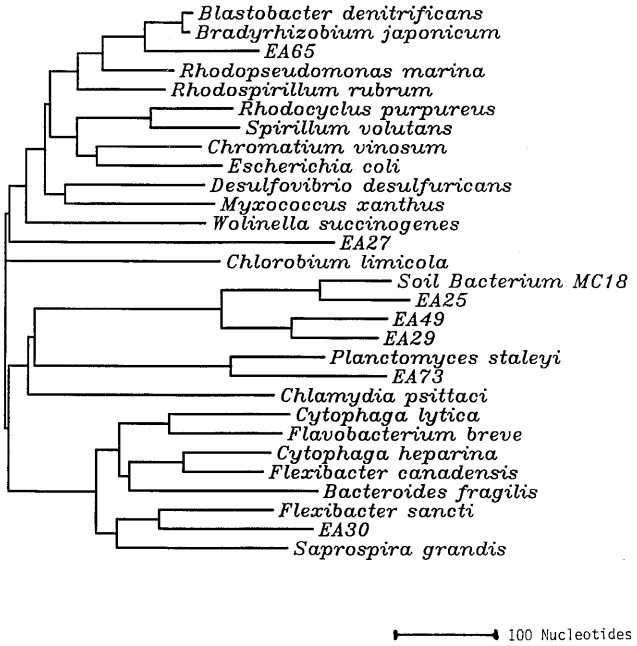


FIG. 1. Phylogenetic tree constructed by a neighbor-joining method of top-scoring sequences from searches of databases for the seven clones obtained in this study.

## RESULTS AND DISCUSSION

**Cloning and sequence analysis of bacterial 16S rDNA.** Seven unique clones, named EA25, EA27, EA29, EA30, EA49, EA65, and EA73, from a clone library containing bacterial 16S rDNA cloned from DNA taken from a natural grassland soil in eastern Washington State, were identified. Approximately 1,300 nucleotides of the 16S rDNA sequences of each clone were sequenced, and the sequences were compared with those in the database (3, 20).

Figure 1 is a neighbor-joining tree (10) of top-scoring sequences from searches of the databases. Four of the seven 16S rDNA sequences analyzed were found to be related to members of the genera *Planctomyces* and *Chlamydia*. Clone EA25 has not been previously cultured on laboratory media and is closely related to strain MC18, found in an Australian soil by similar methods. Environmental clone MC18, representing an organism distantly related to members of the genera *Planctomyces* and *Chlamydia*, was detected from molecular ecological studies of an Australian soil sample by Liesack and Stackebrandt (17) and has not yet been cultured on laboratory medium. The sequence of the 16S rDNA sequence of clone EA25 showed 93% similarity with that of MC18, suggesting that strains similar to EA25 can be widely distributed in soils from temperate regions.

**Strategy for determining the number of copies of EA25 16S rDNA in soil.** Since neither EA25 nor MC18 has been cultivated, enumeration of these strains in soils is currently limited to molecular approaches. We have used a competitive PCR approach developed by Zachar et al. (38) to estimate the numbers of EA25 16S rDNA sequences in Palouse silt loam soil. In this approach, a competitor that consists of a cloned version of the target sequence but has either a deletion or an insertion in the gene of interest must be constructed. The competitor has the same primer binding sites as the target DNA, but its size is different from that of the target. By constructing a competitor with a size different from that of the target, the competitor and target compete for reagents, such as polymerase, primers, dNTP, and Mg, in the same reaction mixture, but their amplification products can easily be distinguished by agarose gel electrophoresis.

After construction of the competitor, a calibration curve is generated by amplifying a range of masses of the target DNA (e.g., EA25 16S rDNA sequences) in the presence of a constant mass of competitor. The logarithms of the ratios of the mass of the amplification product of the target DNA to that of the competitor DNA (log [micrograms target DNA/micrograms of competitor product]) are plotted as a function of the mass of the target DNA. The mass of the target DNA in an unknown sample can then be determined by relating the ratio of the mass of the amplification product of the unknown to that of the target DNA in the calibration curve. The yield of the two products is defined by the following equation: $\log(Nn_1/Nn_2) = \log(No_1/No_2) + [n \times \log(eff_1/eff_2)]$ (28), where $Nn_1$ and $Nn_2$ are amplification product concentrations, $No_1$ and $No_2$ are initial template concentrations, $n$ is the PCR cycle number, and $eff_1$ and $eff_2$ are efficiencies of template amplification. If the efficiencies of amplification of the two templates are the same ($eff_1 = eff_2$), the ratios of products ($Nn_1/Nn_2$) following any cycle ($n$) of PCR amplification depend directly on the ratio of the concentrations of the initial templates ($No_1/No_2$) present. This equation is valid, assuming that the $eff_1/eff_2$ ratio is a constant value even if the efficiencies of amplification of these two templates are not equal ($eff_1 \neq eff_2$).

A number of preliminary experiments must be conducted before this approach can be used to determine the number of copies of EA25 16S rDNA sequences in a soil sample. Most quantitative PCR approaches, including the one used in this study, require an a priori knowledge of the relative rates (or efficiencies) of amplification of the target sequences and the

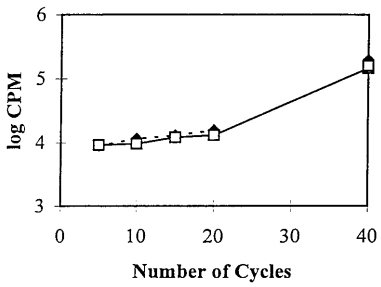


FIG. 2. Amplification rates of pEA25 (◆) and pEA25-C (□).

competitor. In addition, rates of amplification of DNA isolated from soil can be inhibited because of the presence of trace amounts of humic materials that can copurify with the DNA. Comparisons of amplification rates between DNA isolated from soils and those isolated from laboratory cultures must account for any inhibition.

QPCR is additionally complicated in our study since the target sequence is present only in DNA extracted from soil and is not found in pure cultures of the organism. For this reason, the target used for construction of the calibration curve must be a cloned version of the EA25 16S rDNA, designated pEA25. The relative rates of amplification of soil EA25 and pEA25 must therefore be known, and any differences in amplification rates between EA25 and pEA25 must be incorporated into the final calculation of the EA25 16S rDNA copy number.

The efficiencies of lysis, extraction, and purification of EA25 DNA from soil must also be known so that the number of EA25 16S rDNA genes per gram of soil can be determined.

Once these factors have been determined and the assumptions have been checked, the number of EA25 16S rDNA copies can be determined as follows: number of EA25 16S rDNA copies per gram = $Q/(L \times R \times A \times C)$, where $Q$ is the concentration of EA25 from QPCR, $L$ is the lysis efficiency, $R$ is the recovery efficiency, $A$ is the ratio of the amplification rate of pEA25 to that of EA25, and $C$ is $5.98 \times 10^{-18}$ g per copy of pEA25.

**Construction and evaluation of QPCR competitors.** Primers specific to the 16S rDNA of *E. coli* and EA25 were designed and synthesized for these studies, and the specificities of the primers for EA25 were confirmed by sequencing amplification products from soil (data not shown). In addition, we determined that the amplification efficiencies of the target DNA (pEA25) and its competitor (pEA25-C) are the same (Fig. 2).

**Amplification rates of DNA recovered from soil and from laboratory culture.** Soil organic carbon frequently copurifies with DNA in many soil DNA procedures, and humic acids have been shown to be inhibitors of the PCR (31). Inhibition of the PCR of EA25 16S rDNA extracted from soil would result in underestimation of its true numbers if the inhibition were not corrected for. The inhibition of amplification would result in amounts of product from the target smaller than those from the competitor being formed after a given number of cycles.

To ensure in a quantitative manner that the selected primers produced the desired products in soil extracts, the amplification rate of *E. coli* genomic DNA added to soil and that of EA25 isolated from soil were compared with that of DNA isolated from laboratory cultures. We selected approximately the same concentrations of the two templates and amplified each for 40 cycles. Figure 3A shows that with increasing cycle number, *E. coli* genomic DNA that had been purified from soil amplified at the same rate as that obtained directly from laboratory culture. This demonstrates that the PCR amplification of *E. coli* DNA was not significantly inhibited by trace amounts of organic contaminants that might have been present and suggests that amplification of EA25 DNA from soil was also not inhibited. This assumes that amplification rates of DNA from bacteria grown in laboratory growth media are similar to those of DNA from bacteria indigenous to soils, and that any PCR inhibitors that would affect the amplification rates of DNA from indigenous soil organisms would also affect the amplification rates of DNA added to soil. This is likely a safe assumption, since DNA added to soil prior to lysis would be subject to many, if not all, of the same environmental factors (e.g., adsorption to particulates, cations, humic substances, and lysis reagents) most likely to influence the amplification rates of DNA released from indigenous bacteria upon lysis.

The amplification rates of EA25 in soil and pEA25 from laboratory culture are shown in Fig. 3B. The amplification rate

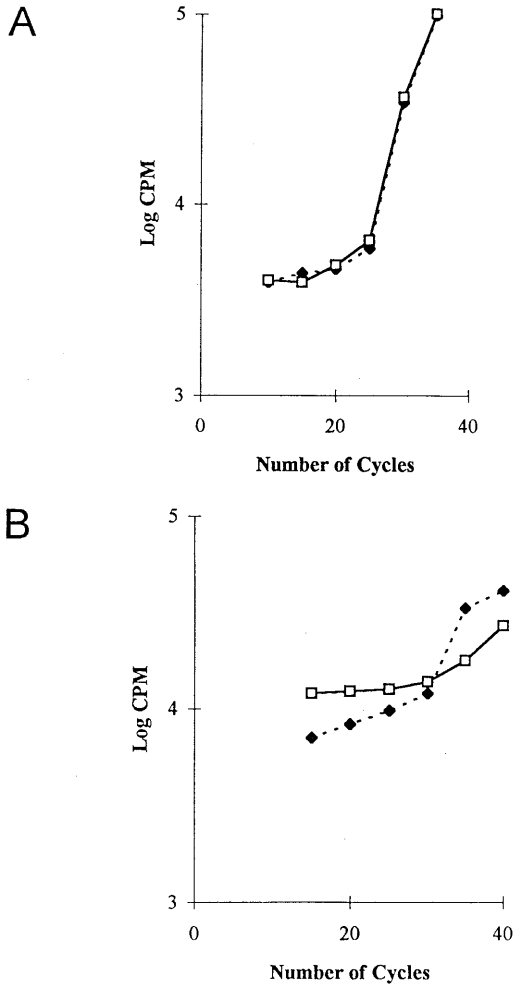


FIG. 3. Amplification rates of template DNAs. (A) Amplification rates of *E. coli* genomic DNA purified from pure culture (◆) and *E. coli* DNA added to soil DNA (□); (B) amplification rates of pEA25 (◆) and EA25 in soil (□). PCR products were labeled by incorporation of $^{32}P$ during the PCR. The relative amounts of products were determined by excising the appropriate electrophoretic bands and measuring the amounts of radioactivity by scintillation counting. Two replicates of each experiment were performed. Error bars, based on ±1 standard deviation, are smaller than the symbols for the datum points.

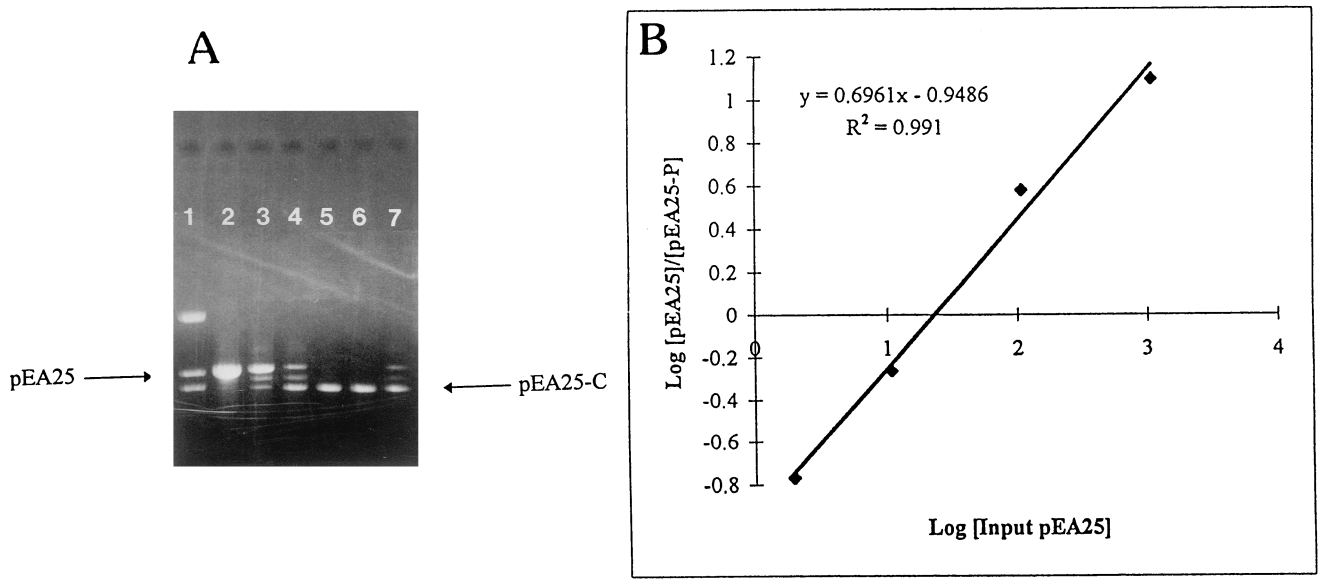


FIG. 4. QPCR of EA25 16S rRNA gene. (A) A range of masses of pEA25 were coamplified with 20.5 pg of competitor (pEA25-C). Lanes: 1, *Bst*I-digested pBR322 as a molecular marker; 2, 1,070 pg of pEA25; 3, 107 pg of pEA25; 4, 10.7 pg of pEA25; 5, 1.07 pg of pEA25; 6, 0.107 pg of pEA25; and 7, 5 μl of soil DNA and 20.5 pg of pEA25-C. (B) Calibration curve of QPCR of EA25 16S rRNA genes. The relative masses of the bands of amplification products corresponding to competitor and pEA25 of the gel in panel A were used to construct this calibration curve. The averages of three determinations are plotted, and error bars, based on ±1 standard deviation, are smaller than the symbols used for the datum points.

of pEA25 was 3.1 times higher than that of EA25 of soil DNA from the beginning of the lag phase through the exponential phase, and the amplification of EA25 did not enter the exponential phase until after 30 cycles of amplification at this concentration. The rather high number of counts per minute associated with the lag phases (approximately cycles 17 through 30) are likely due to unincorporated [$^{32}$P]dCTP that were not completely purified from the bands by electrophoresis. The difference in the amplification rates of the two DNAs is probably due to differences in *rrn* copy number between the genomes of pEA25 and EA25 and/or the proximity of the *rrn* genes to one another (9). The results obtained in this experiment would also incorporate any differences in amplification rates of the two DNAs that might be due to inhibition of the soil DNA by contaminants, even though the amplification of *E. coli* rDNA added to soil was not inhibited. Since pEA25 was used in place of EA25 genomic DNA in the development of the calibration curves for QPCR, a correction factor accounting for this 3.1-fold difference in amplification rates, regardless of the reason for the difference, must be incorporated into the final calculations of EA25 numbers in soils.

**QPCR of *E. coli* 16S rDNA and efficiency of recovery.** Enumeration of any bacterial species by DNA-based methods is a function of efficiencies of cell lysis and subsequent purification of the DNA. We have found that the lysis method used in this study decreases acridine orange direct counts in this soil by 90% (33, 35), and that >99.99% of *Pseudomonas fluorescens* in pure culture is lysed by this method (26). Sequence analysis indicates that EA25 is a gram-negative strain, and it is likely that EA25 would be efficiently lysed by this approach and that gram-positive strains would be more resistant to lysis. The extent of lysis of EA25 cannot be determined at this time since it has never been cultured, nor is it known at this time how lysis in pure cultures is related quantitatively to lysis in the presence of soils.

Two independent methods to estimate the efficiency of recovery of our soil DNA extraction and purification method were used. For the estimation of recovery efficiency by QPCR, 100 ng of *E. coli* genomic DNA was added to 1 g of soil immediately prior to lysis. After isolation and purification of soil DNA, the amount of *E. coli* DNA in the purified soil DNA extracts was estimated by QPCR (data not shown). The recovery efficiency of our soil DNA isolation and purification method, based on two replicates, was estimated to be 3.68% ± 0.11%. Amplification products in soil to which *E. coli* DNA was not added were not observed, indicating that the primers used were specific to *E. coli* DNA in these samples.

We also determined the recovery efficiency by adding approximately 25 kbp of radiolabeled lambda bacteriophage DNA to the soil. The recovery percentage by this approach, based on three replicates, was 2.86%, which was significantly lower ($P = 0.05$; based on two replicates) than that obtained by QPCR of added *E. coli* DNA (3.68%). The reason for this small difference is not known at this time, although it could be that a small amount of $^{32}$P from the dCTP tail that had been added to the tracer DNA during labeling was released. The release of a small amount of [$^{32}$P]dCTP throughout the experiment would result in smaller amounts of label being recovered following ethanol precipitation during the purification procedure, although preliminary experiments conducted without soil indicated that less than 1% of the label was lost in this manner during the time required for this experiment (18). Both methods indicate that the recovery efficiency by our method is low, although the DNA recovered was pure as judged by uninhibited PCR amplification (Fig. 3A). The greatest sources of loss of DNA during purification occurred upon precipitation of nucleic acids by polyethylene glycol (resulting in the loss of approximately 50% of starting DNA) and upon coprecipitation of humic substances by ammonium acetate, resulting in the loss of more than 35% of the starting DNA (13, 15).

**QPCR of EA25 16S rDNA and estimation of EA25 abundance in soil.** The optimum concentration of the competitor

pEA25-C was determined by amplifying 10-fold dilutions of pEA25-C with the purified soil DNA extracts containing EA25, and the concentration of pEA25-C that resulted in approximately equimolar yields of the two PCR products was selected.

A fixed amount of competitor DNA (20.5 pg, corresponding to $3.53 \times 10^6$ copies of pEA25) was added to 5 μl of the purified soil DNA extracts being analyzed and to a single set of standard samples. An example of a typical agarose gel from these experiments is presented in Fig. 4A. The pEA25 16S rDNA of standard samples ranged from 0.107 pg ($1.79 \times 10^4$ copies) to 1,070 pg ($1.79 \times 10^8$ copies). As the concentrations of target and competitor DNA become similar, a third band, located between the competitor and the target, was observed (Fig. 4A, lanes 3 and 4). This third band is commonly observed in competitive PCRs and is attributed to the formation of heteroduplexes composed of target and competitor amplification products (25). Heteroduplexes are formed primarily during the later amplification cycles (28), and the target and competitor amplification products are affected equally. We were able to resolve the target and competitor electrophoretic bands from the heteroduplex bands during analysis, and the quantitation was not affected by their presence.

After coamplification of EA25 16S rDNA from soil and pEA25-C, the calibration curve was constructed (Fig. 4B). In the case of the purified soil DNA samples, the log (EA25)/(pEA25-C) was −0.299 and the amount of EA25 16rDNA in 5 μl of purified soil DNA extracts was estimated to be 6.50 pg. The total volume of the purified soil DNA extracts was 114 μl, and the total amount of EA25 in the purified DNA extracts is 148.2 pg.

For final estimation of the number of *rrn* copies of EA25 present in the sample, the estimated 3.68% recovery efficiency (assuming 90% lysis) and the 3.1-fold difference in amplification rates between EA25 and pEA25 must be factored in: (0.9)(148.2 pg/g)/(0.0368)(3.1) = 1,169.18 pg/g, or $1.169 \times 10^{-9}$ g/g. Assuming a mass of $5.98 \times 10^{-18}$ g per copy of pEA25, this corresponds to $1.95 \times 10^8$ copies of EA25 *rrn* per g of soil. It is not possible at this time to accurately estimate the total numbers of EA25 cells by this approach, because the *rrn* copy number of EA25 is unknown. *rrn* copy number typically ranges from 1 (6, 27) to 14 (37) in members of the domain *Bacteria*, and so it is likely that EA25 is present at levels of between $1.4 \times 10^7$ to $1.95 \times 10^8$ cells per g.

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