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Quantitative Selective PCR of 16S Ribosomal DNA Correlates Well with Selective Agar Plating in Describing Population Dynamics of Indigenous *Pseudomonas* spp. in Soil Hot Spots

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We used a quantitative PCR method targeting 16S ribosomal DNA using competitive PCR for specific detection of indigenous *Pseudomonas* DNA in soil hot spots. The amount of *Pseudomonas* DNA corresponded to the number of culturable *Pseudomonas* bacteria on Gould's S1 agar. This represents the first use of PCR for quantification of indigenous bacteria in more than one sample of soil.

It is a well-established fact that only a small fraction of environmental bacterial communities can be cultivated by current techniques. As a consequence, doubt has been raised that results obtained with traditional agar plating are representative of the actual processes in nature. Among the methods that have been suggested for studying the unculturable fraction of indigenous bacterial communities, some of the most prominent are based on nucleic acids. Extraction and purification of DNA from soil have come in focus (7, 18), and now commercial kits based on recent techniques are successfully marketed (1).

As a means of quantifying unculturable bacterial populations, competitive PCR (cPCR) is promising. cPCR employs the highly sensitive PCR and bypasses quantification problems caused by differences in the exponential PCR amplification of DNA by using an internal standard (4). The internal standard is a competitive DNA template which shares two primers and thus is coamplified in competition with specific DNA sequences in the sample. Since the lengths of the fragments differ, amplification products from the internal standard and sample DNAs are readily separated on a gel.

Competitive PCR has been used in, e.g., marine environments (14), but soil poses problems, as it is heterogeneous and consists of large amounts of inhibitory compounds, so reproducible results are more difficult to obtain. Lee et al. (13) added 16S ribosomal DNA from strain EA25 to soil and could thereafter quantify it by cPCR with good correlation. Lechner and Conrad (12) compared cPCR with traditional cultivation techniques for estimation of hydrogenase-containing bacteria in one rice rhizosphere sample and obtained approximately the same enumeration result by cPCR and cultivation in a single sample. Hallier-Soulier et al. (6) monitored introduced toluene degraders by CFU counting and cPCR with *xylE* in sterilized soil. They found no clear correlation between CFU counting and DNA. Rosado et al. (15) likewise found varying correlation between a DNA assay (most-probable-number PCR) and CFU counting of introduced bacteria in soil. Hence, only one study (12) has applied it for detection of indigenous bacteria in one soil sample, and no study has provided a comparison between quantitative PCR and traditional cultivation techniques for detection and quantification of indigenous bacterial population dynamics in soil.

Our purpose was to assess the reproducibility and appropriateness of competitive PCR in soil ecosystems for quantification of an indigenous *Pseudomonas* population in a soil hot spot. The hot spots were excised bits of young barley roots submerged in soil and monitored during degradation. This report presents, for the first time, a tight association between the number of culturable bacteria and the DNA of indigenous bacteria in soil.

PCR mixtures. Each PCR tube contained a total volume of 46 μl, with 33.4 μl of twice-distilled water, 4.5 μl of 10× Ampli *Taq* PCR buffer (Perkin-Elmer Cetus, Norwalk, Conn.), 0.2 μl of each primer (in a 0.1 mM solution), 4.5 μl of 1% DNase-free bovine serum albumin (Pharmacia, Uppsala, Sweden), 2 μl of Gene Amp 10 mM deoxynucleoside triphosphate mixture (Perkin-Elmer), 0.2 μl of Ampli *Taq* polymerase (Perkin-Elmer), and 1 μl of template DNA. cPCRs contained 1 μl of internal standard and 1 μl of sample as templates. All primers (Table 1) were Gibco BRL custom primers of desalted purity purchased from Life Technologies, Roskilde, Denmark.

Specific PCR amplification. To ensure PCR amplification specific for *Pseudomonas* DNA, a *Pseudomonas*-specific primer set should be found. Probe PSM_G (2) was reported to be *Pseudomonas* specific, and we checked the specificity of the *Pseudomonas* primer again on 1 August 1998 (11). The second primer was 9-27 (16). This sequence is one of several specific for eubacterial DNA conveniently placed 445 bp upstream of PSM_G, hence giving *Pseudomonas*-specific amplification. In order to test if amplification using these two primers was specific, a panel of Fast Soil DNA (Bio 101, Vista, Calif.)-purified DNAs from seven strains were used for PCR (6 min at 94°C; 35 cycles of 30 s at 92°C, 30 s at 52.5°C, and 1 min at 68°C; 6 min at 68°C;

TABLE 1. Primers used in this study

Primer	Sequence[a]
9-27	5′ GAG TTT GAT CCT GGC TCA G 3′
PSM_G	5′ CCT TCC TCC CAA CTT 3′
Intern1R	5′ **CTG ACT CGA TGC GTA** ACC TAG GCT CAT CTG 3′
Intern 2	5′ **TAC GCA TCG AGT CAG** GAT GAT CAG CCA CAC 3′

[a] Sequences in boldface are "sticky ends" used for construction of the internal standard.

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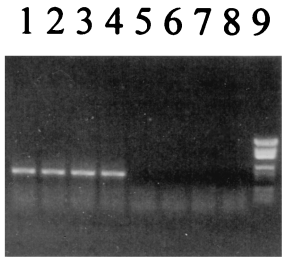

FIG. 1. Test for specific PCR amplification with PSM_G as a *Pseudomonas*-specific primer. Lanes: 1, *P. aeruginosa*; 2, *P. putida*; 3, *P. fluorescens* I; 4, *P. fluorescens* V; 5, *V. vulnificus*; 6, *A. hydrophila*; 7, *C. glutamicum*; 8, no template DNA; 9, pGEM ladder (fragment sizes [from the top], 2,645, 1,605, 1,198, 676, 517, 460, 396, 350, 222, 179, 126, and 75 bp).

and final cooling at 4°C). The PCR tubes were placed in the PCR machine after the temperature had reached 94°C. The organisms used were *Pseudomonas aeruginosa* DSM50071, *P. putida* DSM50208, *P. fluorescens* biovar I DSM50090, *P. fluorescens* biovar V DSM50148, *Vibrio vulnificus* DSM10143, *Aeromonas hydrophila* DSM30016, and *Corynebacterium glutamicum* DSM20300. The *V. vulnificus* strain has 16S sequences closest to PSM_G (differing in 2 of 15 bases). The amplification proved specific for the strains tested (Fig. 1).

Construction of an internal standard. In order to get an internal standard, a shorter fragment with the same primers in the end was constructed as described by Hallier-Soulier et al. (6). Essentially, two primers within the fragment spanning 9-27 and PSM_G in *P. aeruginosa* DSM50071 were constructed (Intern1R and Intern2) (Table 1). The primers also had a 15-bp overlapping region, where the sequences were complementary. The two outer fragments were amplified (9-27 plus Intern1R and Intern2 plus PSM_G), and the products were mixed and PCR amplified with 9-27 and PSM_G as primers. Gel electrophoresis confirmed the presence of one band of the correct size, and the remaining PCR product was purified by the use of a QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) as described by the manufacturer. Tenfold dilutions of the internal standard were prepared and amplified with DNA from *P. aeruginosa* DSM50071. Products of the correct sizes were made, indicating that the cPCR system worked. DNA from a soil *Pseudomonas* strain, JAJ137 (8), was purified

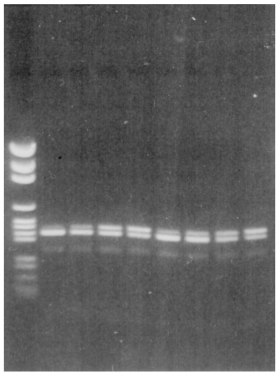

FIG. 2. Example of a gel with products of a cPCR. Lanes: 1, pGEM ladder (fragment sizes, 2,645, 1,605, 1,198, 676, 517, 460, 396, 350, 222, 179, 126, and 75 bp); 2 to 5, twofold dilution series with sample +B, day 3; 6 to 9, twofold dilution series with sample +B, day 6.

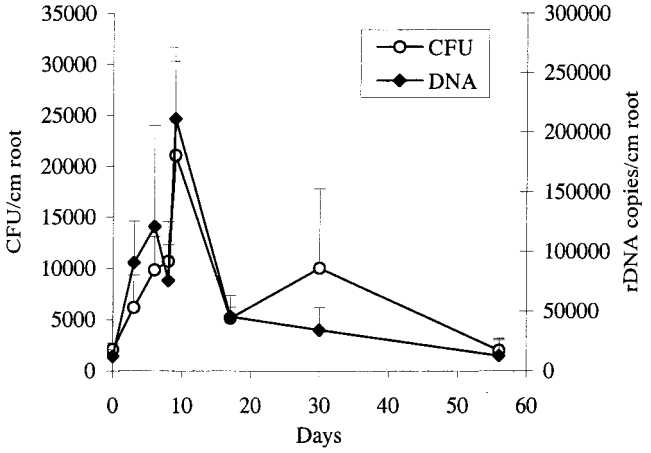

FIG. 3. Comparison of numbers of CFU on Gould's S1 agar specific for *Pseudomonas* and copies of PSM_G-amplified DNA (assuming 100% extraction efficiency from the soil) specific for *Pseudomonas*. Error bars represent standard deviations. Samples are excised bits of young barley roots submerged in soil and monitored during degradation. Soil was incubated at 10°C for 56 days, and agar plates were incubated at 20°C for 3 days (17). rDNA, ribosomal DNA.

by Fast Soil DNA and mixed with the internal standard in different mixtures as described by Suzuki and Giovannoni (16). The ratio of JAJ137 DNA to internal-standard DNA was the same in the PCR products as in the template (data not shown). This suggests that the relative efficiencies of amplification of the template and the internal standard were the same (16). DNA was quantified (A_{260}) on a Shimadzu UV-240 Graphicord UV–visible-light recording spectrophotometer.

DNA purification from soil samples. The Fast Soil DNA purification kit was used in accordance with the manufacturer's instructions. However, cell lysis by the Fast Prep machine was replaced by three freeze-thaw cycles with 15 s of vortexing after each thawing. Samples likely to represent high and low levels of *Pseudomonas* DNA in the sample set were then subjected to PCR with a 10-fold dilution of the internal standard to find the appropriate DNA level range. Twofold dilutions of the internal standard were made and used as competitive template DNA in the PCR tests. An example is shown in Fig. 2. Results for four samples per sampling day were assessed, and average values for each day are shown in Fig. 3 (assuming 100% efficiency of DNA extraction from the soil). The numbers of culturable *Pseudomonas* CFU on Gould's S1 agar, which is known to be *Pseudomonas* specific (5, 9, 10), are also depicted in Fig. 3 (17). The two methods give strikingly similar results. Considering all of the soil samples tested during the 56 days point by point, there is a coefficient of correlation (r^2) between CFU numbers and DNA amounts of 0.60, corresponding to a level of significance of $P > 0.001$ in a product moment correlation coefficient analysis (3). Thus, our results demonstrate the ability of a competitive PCR to quantify DNA in soil reproducibly, even though both the DNA content and the number of culturable bacteria only varied within approximately 1 order of magnitude. Furthermore, the results show that culturability dependent methods, in some cases, are as suitable as molecular methods for describing soil microbial ecology.

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