molecular analysis

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Dispelling the Myths: How Well Do Multiple Competitive PCR Products from 16S rDNA Reflect the Structures of Microbial Communities?

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Profiling microbial communities by PCR-based cloning-free methods targeting 16S rDNA to identify the numerically dominant species in ecologically important habitats has grown rapidly in popularity since its inception in 1993.[9] The question of how well such profiles reflect the relative abundance and cell densities of the target organisms has been tackled several times with encouraging results. Muyzer *et al.*[9] demonstrated that dilution of template to extinction did not affect the patterns of major bands. Felske *et al.*[2] used real-time PCR and thermal gradient gel electrophoresis (TGGE) to demonstrate that the relative abundance of products in complex mixtures did not change dramatically during amplification. Using DGGE, we have recently shown similar results with the analysis of constructed communities inoculated into a microbially diverse topsoil.[11] However, these and other studies have failed to convince many workers that the PCR reaction is not fundamentally flawed when used to analyze complex template mixtures. A commonly voiced opinion, but one that is rarely published, is that the behavior of the PCR is too unpredictable for the results to be accepted in the absence of other supporting data. In this study, we report the use of the DCode™ system in DGGE mode to evaluate a competitive standard included in samples of known and unknown composition, demonstrate the use of this method to count known numbers of cells in laboratory mixtures, and estimate the errors induced when attempting to count *Desulfovibrio vulgaris* cells inoculated into a complex and previously uncharacterized environment. The 16S rDNA copy number per genome is currently unknown for any of the test cells used here, as is the case for uncharacterized environmental organisms.

Materials and Methods

Construction of Standard. DGGE, TTGE and TGGE separate molecules by differences in their melting point. Consequently, we chose as a standard (for use with the PCR primers described in reference 9), 16S rDNA from the X-endosymbiont of the psyllid *Anomoneura mori*,[4] which has a lower melting point than any 16S rDNA molecule detected in environmental samples aside from insect symbionts. The fragment of interest was amplified using the appropriate primers and cloned into PCR II-TOPO™ (Invitrogen). The plasmid was linearized by cutting in the vector sequence with restriction endonuclease *Eco*R I, and quantified by DNA dye-binding fluorescence (VersaFluor fluorometer, Bio-Rad) prior to use.

Cell Counting. The following species were grown as described previously:[11] *Pseudomonas aeruginosa* FRD-1, *Shewanella putrefaciens* 200, *Sphingomonas aromaticivorans* B0695, *Alcaligenes eutrophus* CH34, and *Desulfovibrio*

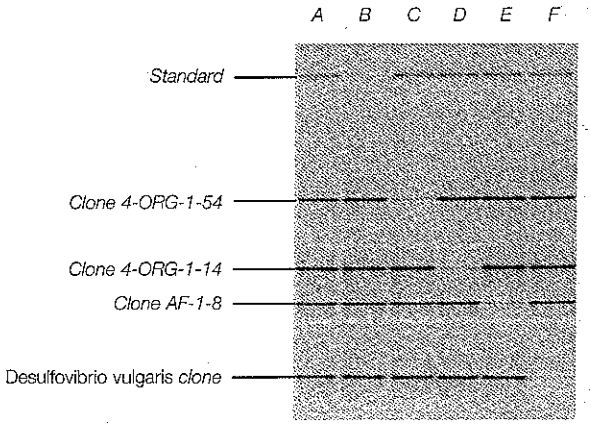

Fig. 1. Multiple competitive PCR products of cloned 16S rDNA fragments analyzed by DGGE after 35 cycles. Lane A, equal starting proportions of all five 16S rDNA templates; lanes B–F, starting amount of one template (in B, the standard; C, clone 4-ORG-1-54; D, clone 4-ORG-1-14; E, clone AF-1-8; F, D. vulgaris) was reduced to 2% of template mixture; equal starting proportions of the other templates made up the remaining 98%. PCR products were detectable from each of the component templates, even when reduced to 2% of the initial template mixture. Amplification efficiencies of templates at higher concentrations did vary slightly, the most obvious bias being against the standard (recovery of which was slightly less than half of the expected proportion at all starting concentrations).

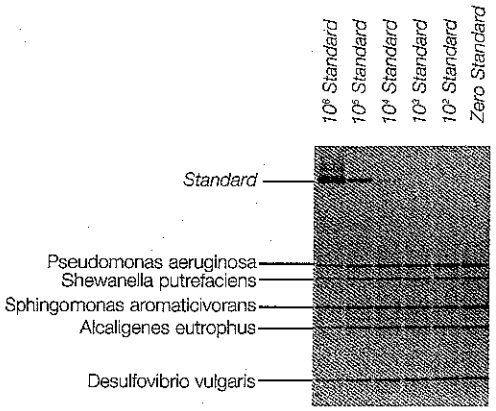

Fig. 2. DGGE analysis of multiple competitive PCR products from mixed genomic DNA spiked with known quantities of standard after 35 cycles of amplification. Estimation of cell numbers from the 10^5 spike generated values approximately 2-fold higher than that expected by microscopic counting prior to cell lysis. The lower recovery of the standard was compensated for by multiplying its signal 2.3-fold prior to band intensity comparison.

vulgaris Hildenborough. Cells were counted microscopically following Acridine Orange staining by three researchers and the results averaged.

DNA Extraction, PCR and DGGE. Extraction of DNA from cultured cells, compost and seeded compost by bead-milling in the presence of CRSR Red (Bio 101) has also been previously described,[11] except that in this study, 0.5 ml of pooled stationary-phase culture or 0.25 gm of compost were substituted for soil. PCR was for the number of cycles specified in the figure legends using the parameters described in Muyzer *et al.*[9] and a Gene Cycler thermal cycler (Bio-Rad) with 1.25 µl Expand™ polymerase (Roche). DGGE was performed using a DCode system (Bio-Rad) with a 1.5 mm thick, 8% acrylamide/bis-acrylamide gel (37.5:1, Bio-Rad) in 0.5x Tris-acetate-EDTA buffer (TAE) between 15 and 70% denaturant concentration at a constant temperature of 60 °C for 16 hr at 40 V. Staining and destaining of gels have been described in reference 7.

Image Analysis. Gels were imaged using the Gel Doc® 1000 system (Bio-Rad), and band intensities were measured using the accompanying software.

Results and Discussion

An initial experiment compared the efficiency of amplification of the standard fragment to several cloned 16S rRNA genes under multiple competitive PCR conditions for 35 cycles (Figure 1). These experiments revealed minor differences in amplification efficiencies among the target molecules, but the difference did not exceed 4-fold for any pair of molecules. In the second experiment, for each of the five cell types, the number of cells, inferred from relative band intensities of the standard relative to each cell-derived PCR fragment, was compared to the number established using microscopy before cell lysis. This study demonstrated that comparison of the band intensities generated an error approximately 2-fold too high by comparison to actual cell counts in this highly characterized system (Figure 2). In the following experiment, *D. vulgaris* cells were added to compost at 5 x 10^8/gm prior to DNA extraction. Aliquots of the extracted DNA were mixed with varying copy numbers of the standard fragment prior to PCR amplification and DGGE analysis.

The results of one analysis, terminated after various cycles (during log-linear amplification, early plateau, and after 25 cycles of plateau phase), are shown in Figure 3. The ratio of standard PCR product to *D. vulgaris* product remained constant up to 20 cycles. Errors in the counting of cell number, estimated by comparison of the standard PCR-DGGE band to that of the *D. vulgaris* band in this highly complex system, increased with increasing cycle number (Figure 4). After a total of 50 cycles, *D. vulgaris* cell count estimates based on comparison with the standard were 50-fold too high. Such a cycle number is extreme. At more commonly used numbers of PCR cycles, the error was quite reasonable; *e.g.*, at 25 cycles (5 following log-linear phase), the error was approximately 5-fold. These errors were mostly attributable to decreasing intensity of the standard product, which correlated with an increase in the amount of single-stranded DNA. It is possible that the low T_m of this molecule strongly reduced its ability to reanneal faithfully during these nonamplifying cycles. During log-linear amplification, and with prior knowledge of the relative amplification efficiencies of the two molecules in a multiple competitive setting, the error was negligible.

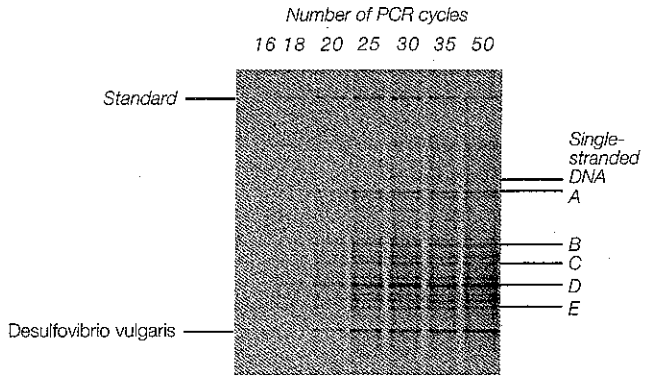

Fig. 3. Quantitative multiple competitive PCR-DGGE analysis of D. vulgaris *spiked into compost. PCR reactions were terminated after the indicated number of cycles prior to DGGE. PCR employed less than 1 ng of template DNA. Other bands represent: A,* Bacillus *sp.; B,* Bacillus *sp.; C,* Bacillus *sp.; D, γ-proteobacterium; E, β-proteobacterium*

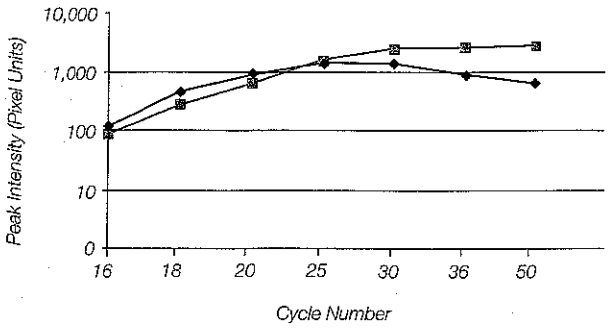

Fig. 4. Changes in band intensities with cycle number. Various exposures of the image in Figure 3 were employed in compiling this data. The ratio of standard to D. vulgaris *products remained constant up to 20 cycles at approximately 1.4 (expected ratio), at which point log-linear amplification of the standard ceased. Errors in the ratio between these products increased to 0.2 after an additional 30 cycles. ◆ indicates the competitive standard; ■ indicates* D. vulgaris.

Considering that even dominant environmental bacteria are typically completely unculturable under current technologies,[3] such errors in the estimation of their numbers are not excessive even if analysis of products during log-linear amplification cannot be guaranteed. Earlier evidence that PCR-DGGE patterns reflect the relative abundance of organisms in a sample is supported. It follows that, if the template number of one sequence is known, those of others can be estimated by image analysis of the DGGE banding pattern. Notably, bands A, B, and C in Figure 3 represented gram-positive organisms (*Bacillus* sp., data not shown), which are generally hard to lyse but were nonetheless easily detected in the presence of more than 10^8 cultured gram-negative *D. vulgaris* cells/gm of sample and in the presence of similar numbers of indigenous proteobacteria.

Clearly, there are problems in using a method such as this in estimating numbers of environmental bacteria. Other than uncertainties over lysis efficiency, additional errors in translating band intensity to cell number are introduced by differing rRNA gene copy number between species.[1] The quality of match between primers and template and the effect of target flanking regions is a further matter of concern in interpreting such data.[6, 10] Furthermore, it is common for an apparently single DGGE band to carry more than one sequence; therefore, band excision and recovery of a legible sequence are required to demonstrate that the product is largely dominated by a single sequence.[5] Nonetheless, results suggest that the behavior of the PCR reaction in a multiple-competitive setting is reliable enough that the effects can be quantified. Reasonable error values can be established to estimate the number of molecules present. The ratio of *D. vulgaris* product to standard under a wide variety of test conditions remained constant at about 2.5, strongly suggesting that correction factors can be established when the aim is to monitor the fate of known organisms or uncultured organisms represented by full-length clones in environmental samples.

Although our knowledge of the vast majority of environmental bacteria consists only of 16S rDNA sequences (*e.g.*, Reference 9 and many others cited therein), this construct and analytical approach may represent a useful quantitative addition to microbial community profiling. Such an approach may be most appropriate when cell growth rates are high, thus minimizing interference from free DNA.[8] With the above qualifications, our results support the contention that profiles of bacterial communities generated by PCR-based methods are a reasonable estimation of dominant *in situ* community structure. The inclusion of an internal standard with unusual denaturant sensitivity, as demonstrated here, provides a quantitative dimension to this method when real-time PCR facilities are not available.

Acknowledgements

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