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Fluorophore-Labeled Primers Improve the Sensitivity, Versatility, and Normalization of Denaturing Gradient Gel Electrophoresis

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Denaturing gradient gel electrophoresis (DGGE) is widely used in microbial ecology. We tested the effect of fluorophore-labeled primers on DGGE band migration, sensitivity, and normalization. The fluorophores Cy5 and Cy3 did not visibly alter DGGE fingerprints; however, 6-carboxyfluorescein retarded band migration. Fluorophore modification improved the sensitivity of DGGE fingerprint detection and facilitated normalization of samples from multiple gels by the application of intralane standards.

Since its advent in environmental microbiology, denaturing gradient gel electrophoresis (DGGE) has become a popular tool for characterizing microbial communities. DGGE and other fingerprinting methods provide advantages of being affordable, relatively easy to use, and amenable to the rapid comparison of multiple samples. Unlike fingerprinting methods that use commercially available size standards, DGGE suffers from a lack of consensus regarding standards for normalization. Gradients formed in different gels are somewhat variable, and without rigorous normalization, gel-to-gel comparisons can be difficult (5, 10). Nonetheless, obtaining fingerprints from various sites and treatments, ideally with replication, usually requires the analysis of multiple gels (5, 6, 8).

Toward solving a similar dilemma, terminal restriction fragment length polymorphism introduces fluorophore labels on one PCR primer, enabling intralane size standards labeled with a separate fluorophore (1). In the context of future innovations for DGGE and temperature gradient gel electrophoresis, Muyzer and coworkers proposed using terminal fluorophores to enable running intralane standards, providing improved sample-to-sample comparisons (11, 12). Ferrari and Hollibaugh indicated that the use of fluorescently labeled primers would eliminate the problem of variable gel staining while minimizing gel handling (5). Hollibaugh and coworkers used fluorescein-labeled primers for DGGE-based studies of oceanic nitrifiers (2, 7), planktonic bacteria (3), and *Archaea* (4). While these studies used this modification, the impact of fluorophore-labeled primers on DGGE fingerprint migration and relative sensitivity remains unknown. Furthermore, running internal standards with DGGE samples has not yet been demonstrated. This report demonstrates that fluorophore-labeled primers provide high sensitivity and increased versatility, since intralane standards improve fingerprint normalization within and between gels.

Methods. Soil samples were taken from Ansio (S4) and Artxanda (S10 and S14) in the Basque Country of Spain during October and November 2002. DNA was extracted using the FastDNA SPIN kit for soil (Q-Biogene, Carlsbad, CA). All PCR amplifications used the same *Bacteria*-specific forward primer, 63f (5′-CAG GCC TAA CAC ATG CAA GTC [9]), with a GC clamp attached at the 5′ end (13), which was purified by polyacrylamide gel electrophoresis (PAGE) by Integrated DNA Technologies (Coralville, IA). The universal reverse primer was 517r, 5′-ATT ACC GCG GCT GCT GG (13). The reverse primer was synthesized alone or with the addition of a 5′-terminal Cy5, Cy3, or 6-carboxyfluorescein (FAM).

Fluorophore-labeled primers were purified by high-pressure liquid chromatography (HPLC). Primers were dissolved in sterile water to a concentration of 100 μM between December 2003 and April 2004 and were stored frozen (−20°C). The PCR amplifications described here were conducted in August 2004. There was no noticeable effect of long-term storage on fluorophore stability. PCR (25 cycles) amplified a ~490-bp 16S rRNA gene fragment from 1 μl of diluted soil DNA (0.625 ng/μl) in a PTC-200 thermal cycler (MJ Research, Waltham, MA), following a previously described protocol (8). PCR products were quantified by UV transillumination on a 1.5% agarose gel stained with ethidium bromide.

DGGE was performed using the Bio-Rad D-Code system (Bio-Rad, Hercules, CA) according to the manufacturer's directions using 6% (37.5:1) polyacrylamide gels with a denaturing gradient of 40 to 70% and a nondenaturing polyacrylamide top-up. Standard markers were generated with equal-volume mixtures of PCR products from 10 16S rRNA gene fragments cloned from cultured isolates or previously run soil DGGE fingerprints (8). For gels in which standards were run with samples, 4 μl (160 ng) of the standard mixture and 100 ng of each soil PCR product were loaded in each lane. Electrophoresis was carried out for 14 h at 60°C and 85 V. After electrophoresis, gels were either imaged directly or first stained with SYBR Green I (Molecular Probes, Eugene, OR) at a 1:10,000 dilution for 1 h and then destained for 15 min in 1× Tris-acetate-EDTA buffer. Gel images were obtained at 100-μm resolution with a Typhoon 9400 variable mode imager (Amersham Biosciences, Piscataway, NJ) or by charge-coupled device image capture from UV transillumination with an AlphaImager 1200 (Alpha Innotech, CA) using a SYBR Green I filter. Typhoon scans were done using the excitation laser and emis-

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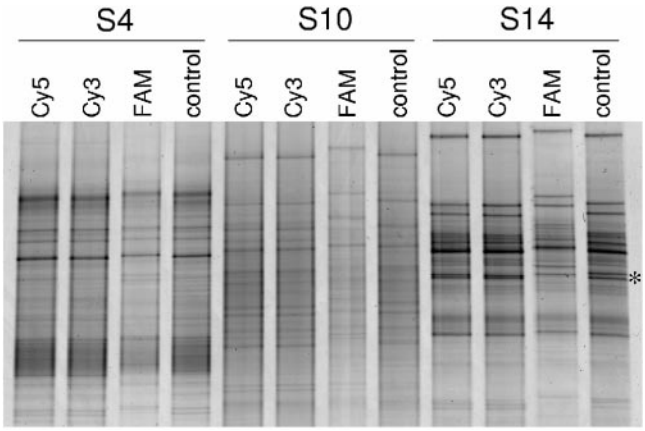

FIG. 1. Impact of fluorophore incorporation on DGGE fingerprints. This SYBR Green I-stained gel contains PCR products from three soils with and without fluorophore labels. An asterisk indicates the position of two gradient-sensitive bands in the S14 fingerprints that appear either singly or as two closely associated bands. This fingerprint variation is not fluorophore specific and simply reflects the challenge of obtaining reproducible fingerprints using DGGE. Instead of 100 ng as for the other samples, only 50 ng of FAM-labeled samples was run in this gel to equalize signal intensities since FAM fluoresces at the same excitation wavelength as SYBR Green I. The amount loaded did not affect fingerprint migration (data not shown).

sion filter recommended by the manufacturer for each fluorophore. All gels were scanned with photomultiplier tube voltages set to maximize signal without saturating fingerprint bands.

Reliability. Three different soil DNA extracts (S4, S10, and S14) were used to determine the impact of Cy5-, Cy3-, and FAM-labeled primers on DGGE fingerprints. Figure 1 shows a gel with DGGE fingerprints stained with SYBR Green I comparing patterns from different fluorophores with a control (no fluorophore label). This gel demonstrated that the fingerprints from Cy5, Cy3, and control samples are almost identical, indicating that these fluorophores had a negligible impact on band migration. Each of the FAM fingerprints was shifted upwards in the gel relative to the other patterns for an unknown reason. Additionally, when gels were sequentially scanned for different fluorophores, faint spectral contamination was observed from both Cy3 and Cy5 fingerprints for FAM scans, whereas scans for Cy3 and Cy5 produced no signal from the other fluorophores (data not shown). As a result, FAM-labeled sample fingerprints should not be compared to other fluorophore-labeled samples or run with Cy3- or Cy5-labeled intralane standards. An appropriate application of FAM is its use as a standard label since spectral cross contamination and uniform alteration of patterns should have no negative impact on the normalization of DGGE gels.

Sensitivity. By preparing dilution series for DGGE with S14 PCR products labeled with either Cy5, Cy3, or FAM or without label, we measured and compared relative sample signal-to-gel background ratios (Fig. 2). A sample gel image is provided (Fig. 2, inset) in which the original unsaturated image intensity was increased to show the fingerprints from the lower sample concentrations. A well-isolated band at the top of the pattern proved useful for quantitative comparisons. Using ImageQuant TL (Amersham Biosciences), the selected band from each dilution was surrounded by a drawn object of constant size for quantification. In order to obtain meaningful estimates of average gel background, 10 band-sized objects were randomly sampled from the background of the gel and averaged.

Using the propagation of error method (delta method) (14), the standard deviation from our gel background average was converted to estimated 95% confidence intervals for sample signal-to-gel background ratios. The confidence interval is $2 \times [(A/B^2)^2 \times (S)^2]^{1/2}$, where A is the band signal; B is the mean background signal; and S is the background standard deviation. Since each sample concentration was run only once, the confidence intervals reflect the variability of the ratio based on background variation (noise).

Cy5-labeled fingerprints provided the highest sample signals relative to gel background with low background noise (Fig. 2). Cy3- and FAM-labeled fingerprints had somewhat lower signal to background ratios than SYBR Green I. The lowest signal to background ratio was observed for unlabeled DGGE fingerprints stained with SYBR Green I and imaged using UV transillumination. This is likely a result of both lower image quality obtained using the charge-coupled device camera and a lower excitation of SYBR Green I under UV light compared to laser scanning at 488 nm. For DGGE, between 100 and 500 ng of environmental PCR products are commonly loaded in each lane. Here we demonstrated high sample signal-to-gel background ratios using Cy5-labeled primers even with only 10 to 100 ng loaded per lane (Fig. 2, inset). Low background increases the detection of less intense bands in complex fingerprints and reduced sample loading facilitates running multiple samples and standards within each lane (see below).

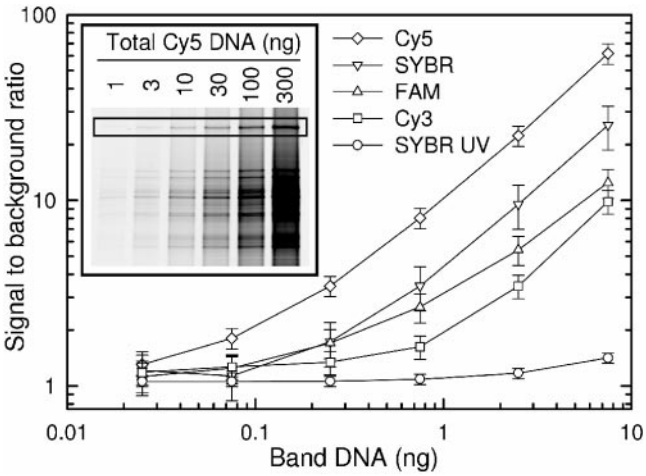

FIG. 2. Comparison of sample signal-to-gel background ratios for fluorophore-labeled and unlabeled PCR product dilutions. Error bars represent 95% confidence intervals for the ratios and were calculated from the standard deviation of the background signals (noise). Unlabeled products were stained with SYBR Green I and detected with either a Typhoon imager (SYBR) or a UV transilluminator (SYBR UV). Inset: Example of a PCR product dilution series (Cy5) indicating the band selected for quantification. This band represents approximately 2.5% of the total fingerprint intensity. While image intensity has been increased for this figure, the original image contains clear patterns for all samples without any saturated pixels.

Normalization. The most significant advantage of using fluorophore-labeled primers is the intralane normalization that is permitted by running each sample with an internal standard. To demonstrate this application, Cy5-labeled PCR products from the three soils were run in triplicate on each of two different gels (Fig. 3A). Both gels were loaded in an identical manner and samples were not run in the middle or outside lanes to enable interlane normalization. FAM-labeled standards were loaded alone in the middle and outside lanes and together with Cy5-labeled environmental PCR products in the remaining lanes (Fig. 3A). The presence of the FAM-labeled standard did not impact sample fingerprints since the addition of as much as 400 ng of FAM-labeled intralane standard (highest amount tested) had no discernible effect on Cy5-labeled fingerprints relative to samples run without standard (data not shown). Cy5-scanned gels were either normalized to the outside and middle lane ladders (interlane normalization) or normalized to all lanes (intralane normalization) using Gel Compar II (Applied Maths, Belgium).

We used a setting of zero optimization in calculating the Pearson correlation values and constructing corresponding unweighted pair group method with arithmetic mean (UPGMA) dendrograms. This ensured that dendrograms differed only on the basis of normalization quality. Figure 3B shows the Pearson correlations between each of the samples using interlane normalization. The dendrogram in Fig. 3C represents the results of intralane normalization. Both dendrograms have similar fingerprint clusters. Replicate samples from different gels clustered separately, with only one exception (S4, Fig. 3B). Replicates from within each gel generally clustered more tightly with intralane normalization.

The average within-gel Pearson similarity value for replicate clusters was 89.2% (range, 78.1% to 97.8%) for interlane normalization and 93.6% (range, 88.5% to 96.9%) for intralane normalization. Tighter clustering of replicate samples with intralane normalization was more apparent with fingerprints collected from gel 2, which was more distorted than gel 1 (Fig. 3A). Furthermore, for each soil sample, the overall six-replicate clusters including both gels were more tightly grouped in the dendrogram generated using intralane normalization (average similarity, 83.7%) than in the interlane normalized dendrogram (average similarity, 81.1%). This is the first reported use of intralane standards with DGGE, and the results demonstrate an overall improvement in gel normalization.

Method evaluation. The fluorophore-labeled primers used in this study were inexpensive. On average, each PCR with a fluorescent primer was US$0.25 more expensive and this cost was largely offset by obviating the time and expense associated with poststaining gels with chemical stain. In addition, by running standards in each lane, additional samples may be added to each gel in lanes formerly occupied by standards. The caveat is that access to an expensive laser-scanning instrument is required, which may limit widespread use of this application at this time. However, in many research institutes such scanners are becoming available.

This simple modification enables additional DGGE versatility, including running DNA- and RNA-derived patterns in the same lane, PCR products from cultured isolates with the original community fingerprint, and confirming excised band PCR products prior to sequencing. The advantages of intralane nor-

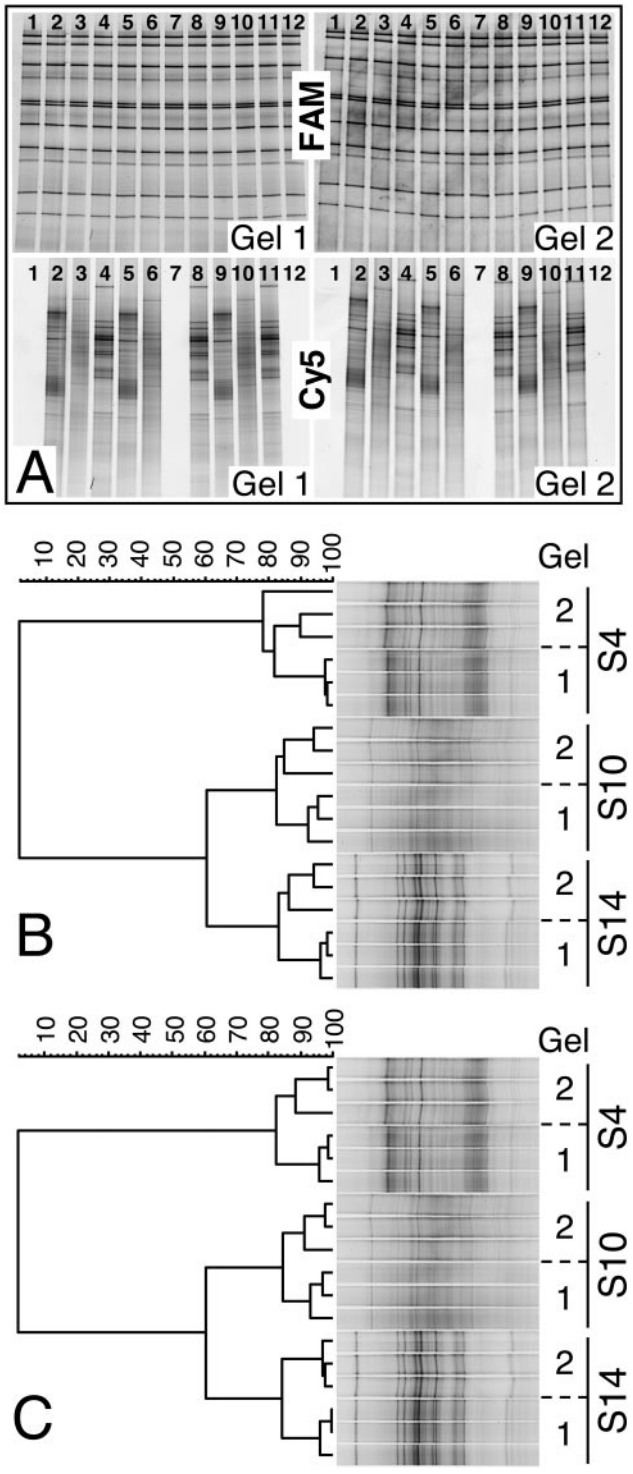

FIG. 3. Comparison of interlane and intralane normalization using fluorophore-labeled standards. (A) Images of duplicate gels scanned for either Cy5 (samples) or FAM (standards). Triplicate samples were arranged in an alternating S4, S10, and S14 order in two gels. (B) UPGMA dendrograms of Pearson correlation matrices generated by interlane normalization. (C) UPGMA dendrograms of Pearson correlation generated by intralane normalization. Fingerprint ordering is identical between the two dendrograms from top to bottom to facilitate comparison.

malization with internal standards as demonstrated here should become more apparent with larger studies involving many samples and multiple gels.

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