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Competitive PCR–DGGE analysis of bacterial mixtures an internal standard and an appraisal of template enumeration accuracy

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Abstract

Analysis of polymerase chain reaction (PCR) amplified 16S rDNA fragments from environmental samples by denaturing gradients of chemicals or heat [denaturing gradient gel electrophoresis (DGGE) and thermal gradient gel electrophoresis (TGGE)] within polyacrylamide gels is a popular tool in microbial ecology. Difficulties in acceptance of the technique and interpretation of the results remain, due to its qualitative nature. In this study we have addressed this problem by the construction and evaluation of a quantitative standard for incorporation into test DNA samples. The standard was based on a naturally occurring 16S rRNA gene carried by the X-endosymbiont of the psyllid *Anomoneura mori*, a γ-proteobacterium. This sequence is the most AT-rich 16S rDNA gene recovered from any cultured organism or environmental sample described to date, and a specifically amplified rDNA fragment denatured under exceptionally low stringency denaturing conditions. The native sequence was modified to incorporate perfect matches to the PCR primers used. The efficiency of amplification of this standard in comparison to a range of 16S rDNA sequences and the errors involved in enumerating template molecules under a range of PCR conditions are demonstrated and quantified. Tests indicated that highly accurate counts of released target molecules from a range of bacterial cells could be achieved in both laboratory mixtures and compost. © 2000 Published by Elsevier Science B.V.

Keywords: DGGE; Competition; 16S rDNA; Culture-independent enumeration

1. Introduction

The amplification of mixtures of polymerase chain reaction (PCR) amplified 16S rDNA fragments combined with their separation on the basis of melting behavior has been an increasingly popular tool in microbial ecology since its first report in 1993 (Muyzer et al., 1993). This approach to community profiling has now been variously combined with group-specific amplification (Heuer et al., 1997), membrane transfer and hybridization (Stephen et al., 1998), band excision and sequence analysis (Kowalchuk et al., 1997a; McCaig et al., 1999), community

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substrate utilization (Duineveld et al., 1998), phospholipid fatty acid analysis (Macnaughton et al., 1999), and with culture methods (Watanabe et al., 1998). The topic has been covered extensively in two recent reviews (Muyzer and Smalla, 1998; Torsvik et al., 1998). However, there have been a number of reports demonstrating that, with some primer-template combinations, the ratio of amplification products following PCR may not accurately reflect the initial ratio of template molecules in these complex, multiple-competitive PCRs (Wagner et al., 1994; Suzuki and Giovannoni, 1996; Hansen et al., 1998; Polz and Cavanaugh, 1998). Such observations have created doubts over the fidelity of these methods, and a common reluctance to permit the inference of changing bacterial community structure from changes in DGGE banding patterns. A single attempt has been made to incorporate a genuinely quantitative aspect into the thermal separation of PCR amplified products (Felske et al., 1998). These authors employed real-time PCR and a fluorescently labeled PCR primer to define the starting concentration of target molecules within their environmental DNA extracts. While elegant and exquisitely sensitive, and generally supportive of the fidelity of multiple competitive PCR, this approach also requires access to expensive specialized equipment and fluorescent tags. Without such facilities, it is difficult for laboratories analyzing environmental samples from diverse sources to guarantee that the final cycle of PCR amplification falls within the logarithmic phase of target amplification. The project described here carried two aims. Firstly, the construction and testing of a unique and versatile competitor 16S rDNA fragment for use with the PCR–DGGE primer set described by Muyzer et al., (Muyzer et al., 1993). This competitor is intended for general use in the numerical estimation of the most common bacterial components of environmental samples detected by PCR–DGGE analysis. The competitive standard consists of a modified 16S rDNA fragment derived from the bacterial X-endosymbiont of the mulberry psyllid *Anomoneura mori* (Fukatsu and Nikoh, 1998). This fragment contains the highest A + T:G + C ratio of any currently available 16S rDNA sequence (Fukatsu and Nikoh, 1998; Maidak et al., 1999). As a result, this competitor molecule generates a PCR amplification product with an exceptionally low melting point, potentially allowing its clear separation from target molecules indigenous to most environmental samples. The second aim was to determine the level of error involved in the use of this competitor under optimal (log. linear amplification) PCR conditions, after various numbers of cycles of sub-optimal (''plateau-phase'') PCR conditions and with different template concentrations.

2. Materials and methods

2.1. Construction and quantification of PCR–DGGE internal standard

The full-length 16S rDNA gene from the X-endosymbiont of *Anomoneura mori*, cloned into the plasmid vector pT7Blue(R) (Novagen; Madison, WI, USA) was a gift from Takema Fukatsu, National Institute of Bioscience and Human-Technology, Japan (Fukatsu and Nikoh, 1998). It was found that the V3 region of this 16S molecule could not be amplified in competition with any other 16S rDNA molecules tested, presumably due to 5 mismatches with the forward primer (mismatches shown in lower case, 5′–3′):

Forward primer:	CCTACGGGAGGCAGCAG
Reverse complement of target site:	ttcAaGaGAGGCAGCAG

The sequence of the reverse primer was ATTACCGCGGCTGCTGG, which had no mismatches to the target sequence. The V3 target region was therefore modified as follows. The target region of X-endosymbiont DNA was amplified by PCR with unclamped primers as described in Muyzer et al. (1993), using 1 unit of Expand HF polymerase, the supplied buffer (Boehringer Mannheim, Indianapolis, IN, USA) and 10 pmol each primer in a total volume of 25 μl on a ''Robocycler 96'' thermocycler (Stratagene, LaJolla, CA, USA). PCR products were extracted using a ''Gene-Clean'' kit (BIO-101; Vista, CA, USA) and cloned as described below. Cloned inserts were re-amplified using the vector primers ''M13'' reverse and ''T7'' according to manufacturer's instructions [Invitrogen; 35 cycles of 94°C (60 s), 55°C (60 s) and 72°C (45 s)]. These products were

re-amplified with the primers described in Muyzer et al. (1993) for screening by DGGE. One plasmid was selected for sequence analysis to confirm the priming-site modification and absence of amplification errors. Double-strand sequencing of plasmid DNA carrying the modified X-endosymbiont rDNA fragment was performed with both vector primers (described above) using an Applied Biosystems automated sequencer (model 373) with ''Prism'' dye terminators (Applied Biosystems, Fostercity, CA, USA). PCR products from this plasmid were obtained using the vector primers, providing a construct carrying the target sequence with 70 bp of flanking region on each end, purified using a ''Gene-Clean'' kit (Bio-101), and quantified fluorimetrically for use in all later competitive studies. This construct is referred to as the ''standard'' throughout.

2.2. Preparation of cloned 16S rDNA fragments for controlled comparison with the standard

Cloned test-16S rDNA fragments were selected to represent a range of A+T:G+C contents over the amplified region. Three of these were environmentally-derived clones; p4-ORG-1-54 (47.4% A+T), p4-ORG-1-14 (43.7%), pAF1-8 (42.7%) and the fourth was a cloned *Desulfovibrio vulgaris* 16S rDNA molecule (38.3% A+T). All generated amplification products of 192 bp (not inclusive of the GC-clamp; Sheffield et al., 1989). Sizes were derived by complete sequence analysis of the three environmental clones, complete sequence analysis of the modified X-symbiont 16S rDNA fragment and from the published sequence of *D. vulgaris* Hildenborough 16S rDNA (Genbank Accession number M34399).

2.3. Cell culture

Five bacterial strains were grown separately to stationary-phase in batch culture. *Shewanella putrifaciens* 200 (Arnold et al., 1988), *Pseudomonas aeruginosa* FRD-1 (Ohman and Chakrabarty, 1981), and *Alcaligenes eutrophus* CH34 (ATCC # 43123) were grown for 26 h at 23°C in Nutrient Broth with shaking. *Sphingomonasaromaticivorans* B0695 (Balkwill et al., 1997) was grown as above for 48 h. *Desulfovibrio vulgaris* Hildenborough (ATCC # 29579) was grown anaerobically for 72 h in an acetate/lactate medium at pH 7.2 containing (g/l): 2.8 sodium acetate; 2.26 sodium lactate; 0.5 yeast extract; 0.1 ascorbic acid; 0.5 $MgSO_4 \cdot 7H_2O$; 0.5 Na_2SO_4; 0.5 K_2HPO_4; 0.5 NH_4Cl; 0.1 $FeSO_4 \cdot 7H_2O$; 7.0 NaCl; and 0.1 sodium thioglycolate. Bacteria in stationary phase were enumerated by acridine orange direct counts (AODC; Skinner et al., 1952).

2.4. Extraction of DNA, PCR and DGGE

Mixtures of cells containing equal proportions of each species (8.16×10^5 cells each) were generated in a total volume of 500 μl. These mixtures were then lyzed by bead beating and the DNA extracted as described in (Stephen et al., 1999). Varying proportions of this extract were used in competitive reactions with the standard as described below. Compost samples (native or spiked with *D. vulgaris* cells immediately prior to extraction; mixed plant-waste origin, sampled at 44°C, shortly after passing a thermal peak at 72°C. A gift of Merja Itävaara, VTT Biotechnology and Food Research, Finland) were extracted by the same method, the only modification being that 0.25 g of compost, rather than 0.5 g, was used. DNA was extracted from soil and fecal samples by this method without modification. All DGGE analyses were as described in (Muyzer et al., 1993), except that gels consisted of a 10–65% gradient of denaturant (100% denaturant=7 M urea, 40% (vol/vol) formamide; Biorad) using a D-Code system (Biorad) at a constant temperature of 60°C. DNA was extracted from soil (collected at the Cannelton industrial site, monitoring site I20, located 1.5 miles west of Sault ste. Marie, upper Peninsula, MI. Principal contaminant was Cr (III), at 24, 507 and 1971 mg kg^{-1} respectively), and human infant feces (age approx. three months; provided by staff members) by the method described in (Stephen et al., 1999) without modification.

2.5. DGGE band excision and sequence analysis

The central portions of DGGE bands of interest from compost DNA were excised and the DNA reamplified as described in Kowalchuk et al. (1997b). PCR products were extracted using Gene-Clean spin columns (Bio101) and the sequences

derived using primer 519r (Lane et al., 1985) and an ABI sequencing system as described above.

2.6. Plasmid isolation and quantification of test DNA

All plasmids were propagated in *E. coli* TOP10F'™ cells (Invitrogen,) and isolated using a ''Wizard mini-prep'' kit (Promega Corp.). All test 16S rDNA fragments were cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) and recombinant plasmids were isolated with the ''Wizard mini-prep'' kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The cloned fragments were then PCR amplified with flanking plasmid-vector primers ''M13'' reverse and ''T7'' according to manufacturer's instructions (Invitrogen). The PCR fragments were purified with ''Gene-Clean'' spin-columns (Bio101) and quantified with a Hoefer DyNA-Quant 200 Fluorometer and Hoechst H33258 dye binding assay according to manufacturer's instructions (Pharmacia Biotech. Inc, Piscataway, NJ, USA).

2.7. Quantitation of ethidium bromide fluorescence of DGGE bands

Gels were stained with ethidium bromide solution and destained in distilled water as described in Kowalchuk et al. (1997a). Images were captured by use of the Alpha-Imager system (Alpha-Innotech, San Leandro, CA). Intensities of the bands within each lane were quantified using the manufacturer's software. When necessary, several exposures of each gel were taken to allow accurate quantitation of band intensities in lanes containing different loading of DNA without saturation of any bands in the individual lanes counted.

2.8. Comparison of AODC and multiple competitive PCR–DGGE to enumerate cultured cells: Effect of template concentration and cycle number

PCR–DGGE, gel staining and image analysis were carried out as above. Template mixtures consisted of DNA extracted from compost spiked with *D. vulgaris* cells or DNA extracted from mixtures of cultured cells into which known numbers of standard copies were added, as detailed in the legends to Figs. 2a, 3, 4a–c. Using the number of X-endosymbiont rDNA fragments as a known value, the number of each target molecule in the mixture was estimated using the formula:

$$N_{(\mathrm{Target})} = ((I_{(\mathrm{Target})} / I_{(\mathrm{Standard})}) \times N_{(\mathrm{Standard})})$$

where $N_{(\mathrm{Target})}$ = Number of unknown target molecules; I = band intensity and $N_{(\mathrm{Standard})}$ = number of modified standard rDNA templates added to the amplification reaction. Four independent reactions were tested for each multiple competitive reaction.

2.9. Statistical analyses

Averages, standard-deviations, logarithmic conversions and χ^2-test results were calculated with the functions available in the Microsoft Excel©version 5.0a for Power Macintosh™ software package (Microsoft Corp., Redmond, Washington, USA).

2.10. Nucleotide sequence accession numbers

The sequence of the modified X-endosymbiont 16S rDNA fragment used in this study has been submitted to GenBank under accession number AF133206. The nucleotide sequences derived from compost DGGE bands were deposited as AF160222-AF160226.

3. Results and discussion

3.1. Evaluation of standard

There are two important criteria in the design of a quantitative standard for inclusion in competitive PCR analyses (Diviacco et al., 1992). The first is that it must be possible to separate the amplification products of the competitor and target DNAs. The second is that it must amplify with an efficiency approaching that of the target molecules to be enumerated. With respect to the first criterion, Fukatsu and Nikoh (1998) noted that the 16S rDNA sequence of this endosymbiont had an exceptionally high A+T ratio (58.4% of the modified fragment).

We predicted from this observation that 16S rDNA fragments from this organism would denature at unique positions in a denaturing gradient, allowing clear separation of this product from environmentally-derived 16S rDNA fragments. We compared the migration of the modified X-endosymbiont fragment to the products generated from environmental samples available in our laboratories. As can be seen from Fig. 1, the X-endosymbiont rDNA fragment was clearly separated from the amplification products generated from test soils, composts, and human infant feces, supporting the general applicability of this standard in a wide range of settings. Considering the second criterion, while the amplification efficiency of a competitor should ideally be identical to that of the target molecule, the introduction of a correction factor is warranted if the bias can be quantified. Similarly, correction factors are generally used to compensate for differences in the size of competitor and target sequences (e.g. Simon et al., 1992; Chandler et al., 1997; Nicholson et al., 1997). We first counted *D. vulgaris* cells by AODC prior to their addition to compost. DNA was then extracted from the spiked compost and their numbers estimated by competitive PCR–DGGE in reactions spiked with known numbers of the standard fragment (Figs. 2a and b). During log linear amplification, and assuming 100% recovery of *D. vulgaris* DNA and a single 16S rDNA copy cell^{-1}, competitive PCR–DGGE provided an estimate of *D. vulgaris* cells that was 2.5× greater than expected from AODC measurement (Table 1). Applying the same criterion to five of the strong bands representing organisms indigenous to the compost sample provided estimates of their numbers as shown in Table 1. The relationship between *D. vulgaris* numbers and the intensity of the standard band deteriorated during plateau phase.

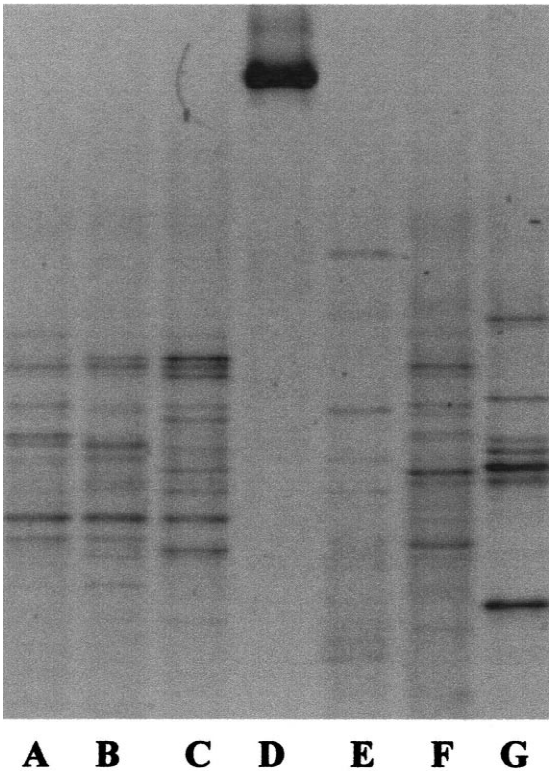

Fig. 1. Separation of standard (modified X-symbiont V3 16S rDNA fragment) from material amplified from complex samples by DGGE. Lanes A–C: Soil contaminated with a mixture of toxic metals, at 0.15, 0.45 and 1.07 m depth respectively. Lane D: Standard fragment. Lane E: Mature compost. Lanes F–G: Human infant feces (two individuals).

3.2. Effect of plateau-phase cycling on estimation of Desulfovibrio vulgaris (Hildenborough) cells in compost

Suzuki and Giovannoni (1996) have previously shown that abundant templates at the onset of amplification can become under-represented following PCR amplification due to inhibition of amplification by self-annealing. The data presented in Figs. 2a and b indicate that at the onset of these experiments, the absolute number of standard molecules was slightly greater than that of *D. vulgaris* 16S rDNA fragments. Therefore, it would be expected that the proportion of standard to *D. vulgaris* amplification products would increase with cycle number, equalizing the ratio between the two products. Although this occurred, both the absolute and relative intensity of the standard product decreased during plateau cycling, a phenomenon which is not accounted for by template-annealing inhibition. This demonstrated that a second effect took place in these reactions, which we assume to be related to the low T_m of the standard fragment. Following log. linear amplification, little or no de novo DNA synthesis occurs, products denature and re-anneal according to their hybridization kinetics. With a higher T_m than the

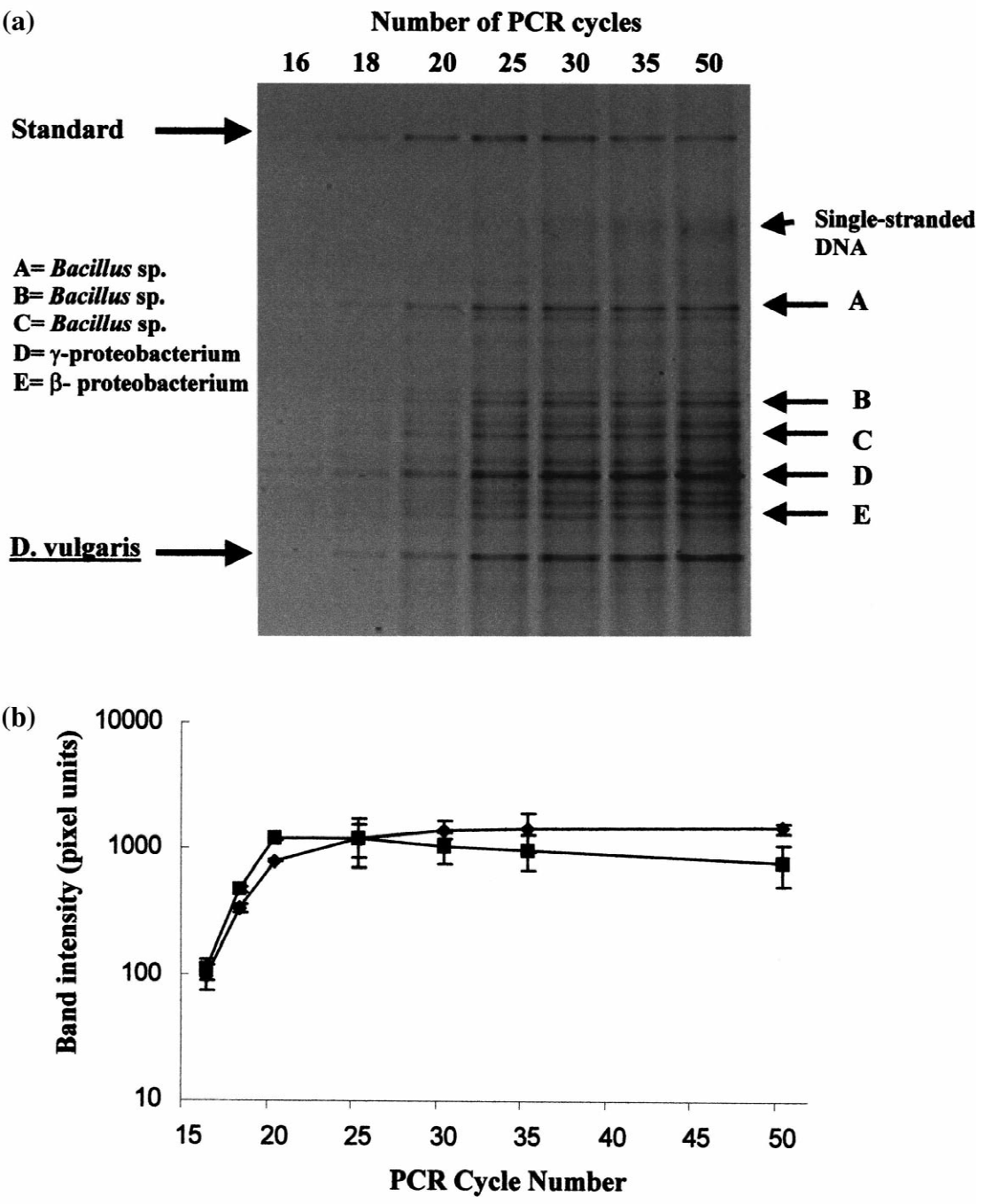

Fig. 2. (a) Quantitative-multiple-competitive PCR–DGGE analysis of *D. vulgaris* spiked into compost. PCR reactions were terminated after the number of cycles indicated prior to DGGE. PCR employed less than 1 ng of template DNA. The effect of PCR cycle number on the ratio of standard to *D. vulgaris* amplification products is shown in Fig. 2b. (b) Changes in the intensities of *D. vulgaris* and standard PCR–DGGE bands with cycle number. Errors represent relative standard deviations on three replicate experiments. *D. vulgaris* bands are represented by (♦), standard bands by (■).

Table 1
Effect of cycle number on apparent ratio of *D. vulgaris* templates to standard templates and estimation of template numbers from dominant indigenous organisms[a]

Templates gram^{-1} compost		16 cycles	18 cycles	20 cycles	25 cycles	30 cycles	35 cycles	50 cycles	Compost source organism
D. vulgaris ($\times 10^3$)	Observed (O)	250	240	240	410	720	770	1760	δ-proteobacterium
template number	Expected (E)	100	100	100	100	100	100	100	–
Ratio O:E		2.5	2.4	2.4	4.1	7.2	7.7	17.6	–
rel. stdev (%)		23	8	3	42	20	35	9	–
Compost 1		–	–	190	–	–	–	–	*Bacillus* sp.
rel. stdev (%)		–	–	19	–	–	–	–	–
Compost 2		–	–	83	–	–	–	–	*Bacillus* sp.
rel. stdev (%)		–	–	8	–	–	–	–	–
Compost 3		–	–	168	–	–	–	–	*Bacillus* sp.
rel. stdev (%)		–	–	16	–	–	–	–	–
Compost 4		–	–	383	–	–	–	–	γ-proteobacterium
rel. stdev (%)		–	–	17	–	–	–	–	–
Compost 5		–	–	135	–	–	–	–	β-proteobacterium
rel. stdev (%)		–	–	35	–	–	–	–	–

[a] Numbers refer to genome equivalents in the initial template DNA added to PCR reactions, which was 0.1% of the total DNA extracted from the spiked mixture. Numbers were calculated from three independent PCR–DGGE reactions.

standard, un-replicated *D. vulgaris* fragments were more likely to re-anneal faithfully. Thus, the combination of de novo synthesis and re-annealing of denatured fragments resulted in a continued increase in *D. vulgaris* rDNA fragments up to 50 cycles, whereas de novo synthesis did not compensate for the failure of standard fragments to re-anneal following denaturation. Loss of standard product was concomitant with an increase in what we took to be single-stranded DNA (Fig. 2a). We investigated this effect further by evaluating the errors induced by amplification of a highly defined mixture of templates.

3.3. Extent of amplification error after 35 cycles in a highly defined multiple competitive reaction

Investigation of bacterial community structures using PCR has been employed in innumerable studies over the past decade. In the vast majority of cases, the number of cycles has not been optimized to terminate prior to the end of log. linear amplification. In general, the highest cycle number used has not exceeded 35. We therefore designed this experiment to estimate the extent of error inherent in extrapolating the results of these studies to representations of community structure, and to relate such any bias to T_m. Representative results are shown in Fig. 3 with the results of combined experiments shown in Table 2. These results demonstrated that 35 cycles of PCR induced significant deviation from the starting template concentration only for the standard fragment, with ratios of the remaining four templates to each other not effected. This may suggest that T_m-induced amplification biases are not significant for fragments with less than approximately 47.4% A+T, the highest value for the sequences tested here, and that errors in the estimation of relative abundance of organisms detected in other studies are not dramatic.

3.4. Effect of relative template abundance and initial template concentration on amplification bias

The validity of DGGE patterns as a representation of the relative abundance of organisms in environmental samples was originally supported by analysis of PCR products generated from template DNA samples diluted to extinction (Muyzer et al., 1993). However, reports using limiting dilutions of template DNA in profiling bacterial communities by PCR are rare (Chandler, 1998). This is most likely because of concerns over the possibility of random drift in amplification events during the early stages of PCR

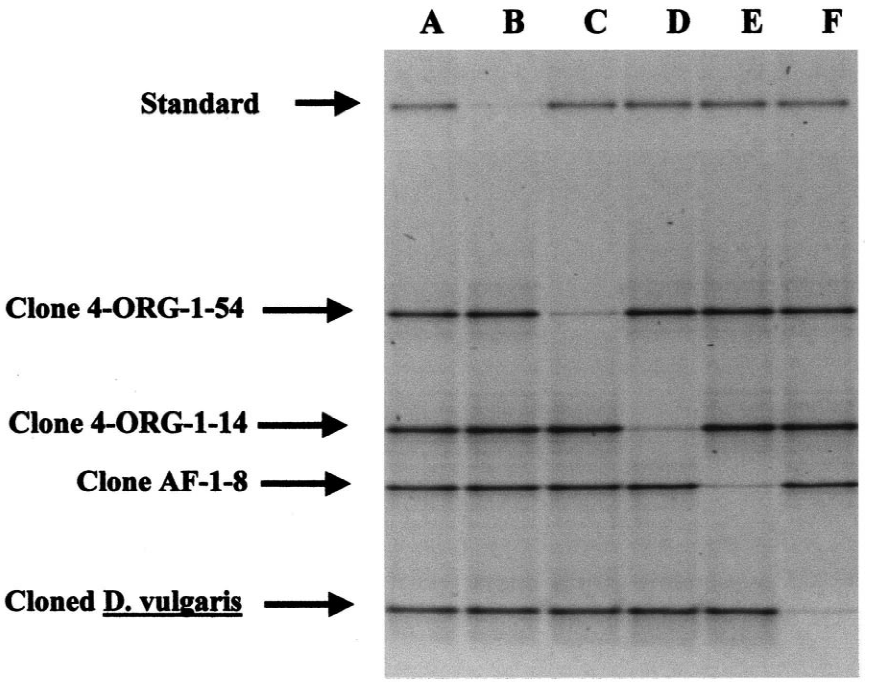

Fig. 3. DGGE analysis of multiple competitive PCR of cloned 16S rDNA fragments. (A) Equal proportions of each clone (B), 2% standard, 24.5% of each other clone (C–F), 2% Clone 4-ORG-1-54, 4-ORG-1-1, AF-1-8 and cloned *D. vulgaris* 16S rDNA, with 24.5% of each remaining clone, respectively. All components of the reaction remained detectable when present at 2% of the initial template mixture after 35 cycles, but the intensity of the standard was below quantitation limits. None of the other components deviated significantly from the expected values after amplification when initially at 2% of the template mixture. Amplification efficiencies of templates at higher concentrations did vary slightly (Table 2).

when highly dilute templates are employed (Wagner et al., 1994; Chandler et al., 1997; Polz and Cavanaugh, 1998). In this study, we diluted a mixture of templates from five bacterial strains spiked with the standard to extinction prior to PCR–DGGE analysis. Fig. 4a shows a multiple competitive reaction using a constant amount of mixed template derived from five cultured organisms (DNA extracted from 1.6×10^4 cells of each strain) in combination with varying quantities of the standard molecule. Table 3 shows the calculated template numbers from reactions spiked with 10^5 and 10^4 standard template copies, and demonstrates that a 10-fold change in standard template copy number and the test template had a negligible effect on quantification. Reactions spiked with 10^6 standard copies generated a standard band approximately 100-fold more intense than the test organisms, and were not used in quantitation. The reaction spiked with 10^5 standard copies generated approximately equal quantities of each template component after 35 cycles and the template for this reaction was selected for dilution to extinction prior to multiple competitive PCR and DGGE analysis (Fig. 4b). The highest dilution producing bands of sufficient intensity to be quantified contained 160 copies of the standard molecule and 26 genome-equivalents from each test organism. Changes in the ratios of the components of this mixture and estimation of initial cell numbers with dilution are given in Fig. 4c. These results showed that template dilution to the use of 130–800 copies of each template slightly improved the congruence between cell number established by AODC and that estimated by comparison of band intensity to that of the standard. Moreover, this template dilution demonstrated the least variation between replicates, although variation was not great at any of the lower dilutions tested. All discrepancies between

Table 2
Amplification errors for 16S rDNA fragments from different template molecules after 35 cycles of PCR[a]

Template	X-Symbiont % total	4-ORG-1-54 % total	4-ORG-1-14 % total	AF1-8 % total	*D. vulgaris* % total
A Observed	8.69 (0.96)	23.94 (2.57)	27.73 (2.38)	19.58 (0.72)	20.06 (1.01)
Expected	20	20	20	20	20
B Observed	0 (0)	26.94 (2.62)	30.56 (2.38)	20.41 (0.63)	22.09 (2.6)
Expected	2	24.5	24.5	24.5	24.5
C Observed	10.94 (1.21)	2.03 (0.91)	39.15 (2.45)	23.2 (0.8)	24.67 (2.06)
Expected	24.5	2	24.5	24.5	24.5
D Observed	10.82 (1.57)	32.86 (2.61)	3.06 (0.97)	25.62 (0.53)	27.64 (1.51)
Expected	24.5	24.5	2	24.5	24.5
E Observed	10.31 (1.16)	29.39 (3.02)	34.14 (0.9)	1.44 (1.04)	24.71 (1.8)
Expected	24.5	24.5	24.5	2	24.5
F Observed	9.96 (1.28)	30.73 (3.84)	34.92 (3.4)	23.1 (0.44)	1.29 (0.92)
Expected	24.5	24.5	24.5	24.5	2

[a] The proportion of X-Symbiont-derived PCR product is significantly different from its proportion in the initial mixture of template molecules before amplification (χ^2 probability of <0.05); proportions of PCR products derived from other template molecules did not show significant amplification bias (χ^2 probability of >0.05). χ^2 values were calculated for each different template molecule on the combined data from PCRs labeled ''A'' to ''F'' in Fig. 3.

these values at all template concentrations were within the known range of 16S rRNA copy numbers cell^{-1} (1–14 *rrn* copies genome^{-1}; Young and Cole, 1993; Cole and Girons, 1994).

4. Conclusions

The findings described in this study support the quantitative nature of 16S rDNA PCR–DGGE profiles of bacterial communities generated with the primers of Muyzer et al., (1993). The standard, based on a 16S rRNA gene derived from the X-endosymbiont of *Anomonura mori*, can provide a useful internal control for use in multiple competitive PCR–DGGE of 16S rDNA from environmental samples. The accuracy of this measurement is dependent on minimizing cycle number and/or template concentration, although extremely low template numbers (<100 dominant cell type^{-1}) are not recommended. Clearly, translating template number in a DNA extract into cell numbers/gram sample is not trivial. The efficiency of lysis of different cell types is likely to be variable, and factors such as rRNA copy-number/genome and genomes/cell must be considered (Farrelly et al., 1995). However, in this study detection of several Gram-positive bacterial strains (*Bacilli*) in compost was not prevented by the addition of a large relative abundance of Gram-*D. vulgaris* cells. Equally, the ratio of *D. vulgaris* product to standard under all test conditions remained constant at ca. 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. The quality of match between primers and template is a further matter of concern in interpreting such data (Polz and Cavanaugh, 1998). For example, the forward primer used here is directed at a site commonly considered sufficiently conserved to define Bacteria in fluorescent in situ and membrane-hybridization analysis (e.g. Amann et al., 1990; Snaidr et al., 1999), yet carried five mismatches to the X-endosymbiont 16S rDNA sequence. Further, it is common for an apparently single DGGE band to carry more than once sequence, therefore band excision and recovery of a legible sequence is required to demonstrate that the product is dominated by a single sequence (Carcia-Delgado et al., 1998). Nevertheless, while our knowledge of the vast majority of environmental bacteria consists only of 16S rDNA sequences (e.g. Felske, 1999), this construct and analytical approach may represent a useful quantitative addition to

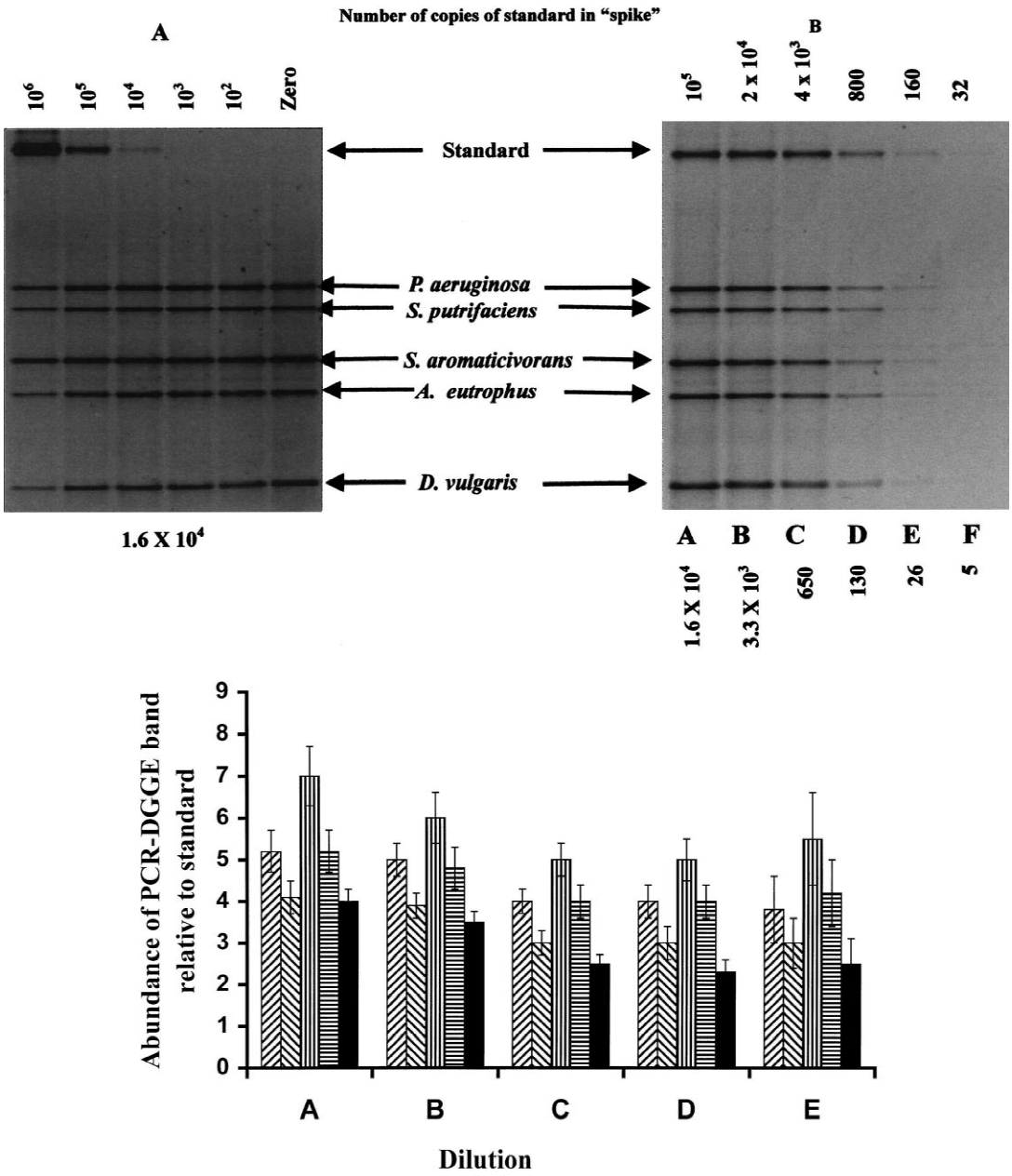

Fig. 4. (a) Multiple-competitive PCR–DGGE analysis of DNA extracted from a defined mixture of cells with varying concentrations of the standard. All reactions contained 1.6×10^4 genome-equivalents of each member of the bacterial mixture, and the standard was varied between 10^6 and zero copies. (b) Multiple-competitive PCR–DGGE analysis of defined template diluted to extinction. The same template used in Fig. 4a, second lane from left, was diluted in 5-fold steps prior to PCR–DGGE analysis. Numbers of standard copies per reaction are given above the image, numbers of genome equivalents of each test-organism below. Lanes are labeled A–F to assist in interpretation of Fig. 4c. (c) Quantification of changes in signal from test organisms relative to standard copy with initial template concentration. Ratio of each test cell to standard construct remained constant at 6.2. Datasets A–E represent the mixtures shown in Lanes A–E of Fig. 4b. Bars represent relative standard deviations on three replicate experiments. Patterns represent *P. aeruginosa* (▨), *S. putrifaciens* (▧), *S aromaticivorans* (▥), *A eutrophus* (▤), and *D. vulgaris* (■) respectively. Bands in Lane F of Fig. 4b were too faint to be quantified accurately.

Table 3
Effect of standard template:test template ratio on error values[a]

	Standard	*P. aeruginosa*	*S. putrifaciens*	*S. aromaticivorans*	*A. eutrophus*	*D. vulgaris*
$N = 10^5 \times$ Standard copies (modified X-symbiont 16S rDNA fragment)						
PCR–DGGE band intensity relative to that of standard	1	0.845 (0.09)	0.68 (0.1)	1.144 (0.155)	0.648 (0.099)	0.65 (0.092)
Calculated template copy number		84502	68001	114402	84800	65002
Expected template copy number (cell number)		16320	16320	16320	16320	16320
Ratio of calculated:expected copy number		5.2	4.1	7.0	5.2	4.0
$10^4 \times$ Standard copies (modified X-symbiont 16S rDNA fragment)						
PCR–DGGE band intensity relative to that of standard	1	8.8 (1.2)	7.3 (0.3)	11.4 (1.1)	6.8 (0.6)	7.1 (0.3)
Calculated template copy number		88002	73002	114001	68001	30870
Expected template copy number (cell number)		16320	16320	16320	16320	16320
Ratio of calculated:expected copy numbers		5.4	4.5	7.0	4.2	1.89

[a] Numbers in brackets indicate the standard deviations of the original band intensities ($n = 3$).

microbial community profiling, and may be adaptable for use with other primer sequences (e.g. Marchesi et al., 1998). Such an approach may be most appropriate when cell growth rates are high, thus minimizing interference from free DNA (Lorenz and Wackernagel, 1987). 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.

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