

# Denaturing Gradient Gel Electrophoresis Can Fail to Separate 16S rDNA Fragments with Multiple Base Differences

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## Abstract

Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rRNA gene fragments is commonly used to examine natural bacterial communities. However, recent studies have reported difficulty in separating different 16S rDNA sequences by DGGE. We utilized site-directed mutagenesis to create *Escherichia coli* 16S rRNA gene fragments differing by 1-4 base pairs, and examined the migration of these fragments in DGGE gels. DGGE could always separate sequences differing by a single base pair, but multiple sequence differences were not so easily resolved. Two sequences that differed by 2 base pairs showed identical migration in DGGE gels and could not be separated in a mixed sample. This limitation should be considered when using DGGE to examine natural bacterial communities.

## Introduction

The usefulness of denaturing gradient gel electrophoresis (DGGE) in the analysis of microbial communities rests on the assumption that different sequences will migrate to different positions in DGGE gels (Muyzer *et al.*, 1993; Jackson and Churchill, 1999). DGGE separates DNA sequences based on their melting behavior (Lerman *et al.*, 1984) and simulations show that 95% of single base sequence differences will be detected by this method (Myers *et al.*, 1985). The ability of the technique to separate genomic sequences differing by more than one base has rarely been examined, although it has been suggested that detection of multiple sequence differences may be more difficult (Lyons, 1994). Indeed, some authors have reported difficulty in separating what were thought to be different bacterial sequences (Kowalchuk *et al.*, 1997; Vallaeyts *et al.*, 1997; Jackson *et al.*, 1998). In a previous study, we observed that two sequences that comigrated in DGGE gels differed in 5% of their base pairs, although they did

share the same GC content, which could account for their similar migration (Jackson *et al.*, 1998). In this study, we demonstrate experimentally that partial 16S ribosomal DNA sequences differing by more than one base pair can migrate to identical positions in DGGE gels.

## Results

Not all sequence changes in 16S rDNA fragments could be detected by DGGE (Figure 1). Single nucleotide differences were always resolved (Figure 1, any adjacent single products), but multiple differences were not resolved as easily. The original sequence and that derived from primer P2B migrated to identical positions (Figure 1, lanes 0 and 2), even though they differed by two nucleotides. The addition of a third nucleotide change resulted in an easily detected difference in migration, but sequences differing by four nucleotides were only slightly separated (Figure 1, lanes 0 and 4). The full strength and the 1/4 diluted samples showed identical migration patterns. The mixed sample, containing all five amplification products, was resolved into four distinct bands, with a denser band appearing where the P2 and P2B amplification products comigrated (Figure 1, lane mix).

## Discussion

The comigration of different sequences to the same position shows that the assumption that one band equals one genome is not always valid. While this limitation of DGGE is becoming more accepted, it has not been experimentally demonstrated until now. Likewise, the presence of bands at similar positions in DGGE gels does not confirm the presence of the same sequence or bacterial species in each sample. Although these limitations do not prohibit the use of DGGE in comparisons of different microbial communities, they do limit the conclusions that can be drawn from this technique alone. Excision and sequencing of the bands in question is required to confirm that they are identical. This study also demonstrated that genetic similarity does not necessarily confer similar migration properties in DGGE gels. Other authors (Vallaeyts *et al.*, 1997) have observed the lack of correlation between the number of sequence differences and DGGE migration patterns.

Although it might be possible to separate similarly migrating sequences by using a smaller denaturing gradient, the gradient used in this study (30-55%) was smaller than those most commonly used [e.g. 15-55% (Muyzer *et al.*, 1993), 20-70% (Teske *et al.*, 1996), 35-80% (Ferris *et al.*, 1996), 40-70% (Murray *et al.*, 1996)]. The use of small gradients is probably unwise in the analysis of environmental samples, which may contain sequences spanning a broad range of melting

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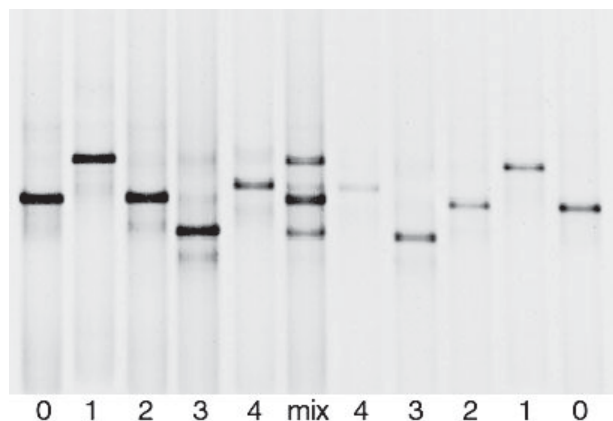


Figure 1. Denaturing gradient gel electrophoresis analysis of five partial 16S rDNA sequences differing from the *Escherichia coli* sequence by 0-4 consecutive base pairs (bp). 0=original *E. coli* sequence, 1=1bp difference, 2=2bp differences, 3=3bp differences, 4=4bp differences, mix=a mixture of all 5 sequences. Samples to the right of the mixed sample are identical to those to the left, but are diluted 1/4. The entire gel contained a 30-55% denaturing gradient, with the area shown in the figure covering approximately 35-40%.

temperatures. In our experience, smaller gradients (<15-20%) also tend to reduce the clarity of bands, making the examination of samples containing multiple DNA sequences difficult.

The artificially modified sequences used in this study were generated by site-directed mutagenesis using degenerate primers, and hence resulted in sequence changes towards the end of the DNA fragment. The melting properties of a DNA sequence are determined not only by its nucleotide composition but also by the interactions between different nucleotides within the molecule (e.g., nearest neighbor interactions, Breslauer *et al.*, 1986, Sugimoto *et al.*, 1996). Sequence changes away from the ends of the DNA fragment may not result in the same changes in DGGE migration as those demonstrated in this study. Such internal sequence differences are likely to be more common when examining natural bacterial communities, although comigration of different sequences is still likely. Others have reported difficulty in separating 16S rDNA amplification products from environmental bacteria that were known to be different (Kowalchuk *et al.*, 1997; Vallaeys *et al.*, 1997; Jackson *et al.*, 1998).

In summary, although the analysis of 16S rDNA fragments by DGGE has great value in environmental microbiology, it does have limitations. Perhaps the best

use of DGGE in the examination of microbial communities is as an initial means of comparing two or more environmental samples. If the samples show different banding patterns, then the bacterial communities they contain are undeniably different. However, if they show a similar banding pattern, then they may or may not contain similar bacterial communities and further analyses are needed.

#### Experimental Procedures

##### DNA Extraction and PCR

DNA was extracted from a culture of *Escherichia coli* BL21 (Novagen, Madison, WI, USA) using the high salt, extended heating method of Zhou *et al.* (1996). A portion of the V3 region of 16S rDNA corresponding to positions 341-534 was amplified using the P2 and P3 primers of Muyzer *et al.* (1993). The P3 primer has a 40 base pair GC-clamp to facilitate melting of the entire region of interest, and the DNA fragment produced has been shown to have just one melting domain (Muyzer *et al.*, 1993). DGGE examination of this region of the 16S rRNA gene has been performed previously in ecological studies (Muyzer *et al.*, 1993; Murray *et al.*, 1996; Jackson *et al.*, 1998). Amplification was performed using reactant concentrations described previously (Jackson *et al.*, 1998). A touchdown procedure (Don *et al.*, 1991) was utilized starting with an annealing temperature of 65°C for four cycles, which was lowered to 63°C for four cycles, 61°C for two cycles and subsequently decreased by 1°C every second cycle until a touchdown at 55°C, with a further eight cycles. Denaturing and extension temperatures were 95°C and 72°C, respectively.

##### Generation of Altered PCR Products

The amplification product obtained was visualized on an agarose gel, and the resulting band excised and purified using standard methods (Sambrook *et al.*, 1989). The purified product from this amplification was used as the template in a second amplification which changed the sequence by 1 bp using site-directed mutagenesis (Higuchi *et al.*, 1986; Kadowaki *et al.*, 1989). Primer P2 was modified by one base to give a new primer, P2A (Table 1), and this replaced P2 in the reaction mixture. Amplification conditions in this mutagenesis reaction were less stringent, with no touchdown procedure and an annealing temperature of 50 °C for 30 cycles. The product of this new reaction was examined on an agarose gel and excised, purified, and used as the template in a subsequent reaction using the original touchdown PCR protocol (with P2A replacing P2). This yielded ample concentrations of a specific amplification product that differed from the original by 1 bp.

This new amplified fragment was used to seed a further mutagenesis reaction (using primer P2B) that changed the sequence by an additional base. This process was repeated utilizing primers P2, P2A, P2B, P2C and P2D (Table 1) to generate a total of five amplification products, with sequences which differed from the original *E. coli* sequence by 0, 1, 2, 3 and 4 bp. All amplification products were sequenced to confirm identity, and to ensure that no other sequence differences were generated through amplification error.

##### DGGE analysis of altered PCR products

PCR products were analyzed in DGGE gels consisting of a 30-55% urea/formamide gradient in a 6% acrylamide gel. Because excessive quantities of DNA might obscure small differences in migration, we also loaded the same samples diluted 1/4. A mixture of all five amplification products (equal volumes each at 1/4 dilution) was also loaded, to represent a mixed sample containing multiple genomes, which would be typical of environmental analyses. Electrophoresis was performed at 130 V and 60°C for 4 h under conditions as otherwise recommended by Muyzer *et al.*, (1996). We had previously determined that 4 h was the optimum electrophoresis time under these conditions.

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Table 1. Amplification Primers Used to Create Different Sequences Through Site-Directed Mutagenesis of *Escherichia coli* 16S rDNA Fragments

Primer Name	Primer Sequence <sup>a</sup>	Cumulative differences from original P2 sequence
P2	ATTACCGCGCTGCTGGCTGG	0
P2A	ATTACCGCTGCTGCTGGCTGG	1
P2B	ATTACCGCTGCGGCTGGCTGG	2
P2C	ATTGCCGCTGCGGCTGGCTGG	3
P2D	ATTGCCGCTACGGCTGGCTGG	4

<sup>a</sup> Sequence differences between each primer and the one preceding it are underlined

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