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Electrophoresis time impacts the denaturing gradient gel electrophoresis-based assessment of bacterial community structure

W.V. Sigler^{a,*}, C. Miniaci^b, J. Zeyer^b

^aDepartment of Earth, Ecological and Environmental Sciences, University of Toledo, 2801 W. Bancroft MS 604, Toledo, OH 43606, USA

^bSwiss Federal Institute of Technology, Institute for Terrestrial Ecology, Grabenstrasse 3, 8952 Schlieren, Switzerland

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Abstract

We investigated the impact of denaturing gradient gel electrophoresis (DGGE) run time on the assessment of bacterial community structure. Results indicated that increased electrophoresis run time (while maintaining 1000 volt-hours) resulted in dissimilar profiles, likely due to instability of the denaturing gradient. We recommend that DGGE run times be minimized to provide optimal band resolution, as extended electrophoresis times can greatly impact subsequent band-based analyses.

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Denaturing gradient gel electrophoresis (DGGE) is a popular method for assessing the structure of microbial communities in environmental samples (Muyzer and Smalla, 1998). The technique is based on the electrophoretic separation of PCR-generated double stranded DNA in an acrylamide gel containing a gradient of a denaturant. As the DNA encounters an appropriate denaturant concentration, a sequence-dependent partial separation of the double strands occurs. This conformational change in the DNA tertiary structure causes a reduced migration rate and results in a DNA band pattern representative of the sampled microbial community. Modern image analysis systems have proven to be of value for the analysis

of DGGE bands and their associated patterns. For instance, pairwise matching of DGGE bands in separate gel lanes has facilitated the calculation of similarity coefficients to describe relationships between communities (van der Gucht et al., 2001; Sigler et al., 2002). Additionally, the use of common diversity indices that incorporate band number and intensity as surrogates for phylotype number (Casamayor et al., 2000) and abundance (Øvreås et al., 1997; Konopka et al., 1999; Nübel et al., 1999; McCaig et al., 2001; Sigler and Zeyer, 2002), respectively, is also popular. However, this application of band information is often limited to less complex systems due to PCR amplification biases including preferential- and nonspecific amplification (reviewed by van Wintzingerode et al., 1997; Suzuki and Giovannoni, 1996) and heterogeneity in *rrn* copy number (Farrelly et al., 1995). Regardless of the methods chosen to interpret banding patterns, key to the success of DGGE-based commu-

* Corresponding author. Tel.: +1-419-530-2897; fax: +1-419-530-4421.

E-mail address: von.sigler@utoledo.edu (W.V. Sigler).

nity structure analysis is the separation of PCR products that results in the optimum resolution of as many potential phylogenetic markers as possible.

As with many molecular methods, the steps involved in DGGE analysis are more or less consistent among differing laboratories, but not standardized. In general, the PCR product length analyzed is between 200 and 600 base pairs (bp). The acrylamide percentage of the gel is commonly either 6% or 8% and most runs are performed at a temperature of 60 °C across denaturant concentrations from as low as 20% to as high as 70% or more (a 100% denaturing solution is defined as 40% [vol/vol] formamide and 7 M urea). However, much inconsistency exists in the choice of electrophoresis volt-hours (V·h), which is a function of applied voltage and running time. This inconsistency is reflected in the applied V·h described throughout the DGGE literature, which ranges from a minimum of 455 V·h (130 V for 3.5 h; Cocolin et al., 2001) to 2100 V·h (100 V for 21 h; Gejman et al., 1998). In preliminary experiments we observed that extended electrophoresis times resulted in sub-optimal band separation and resolution. To further explore the idea that extended electrophoresis times can impact community analyses performed by assessing DGGE band patterns we generated a model microbial community from soil (a 100-year-old soil from Transect 2 of a glacier forefield as described in Sigler and Zeyer, 2002). Triplicate soil samples (5 g each) were shaken in 50 ml of minimal media supplemented with glucose (1 g l⁻¹; Cullington and Walker, 1999) at 23 °C (100 rpm) until the OD₆₀₀ was approximately 0.5. Preliminary studies indicated that the glucose amendment helped to promote an enriched microbial community that ultimately generated a banding pattern suitable for optimizing DGGE conditions. The soil was allowed to settle and the supernatant was transferred to a sterile Falcon tube and centrifuged at 5000 × g for 10 min to pellet the cells. The supernatant was removed, the cells were resuspended in DNA extraction buffer and DNA was extracted, pooled, and quantified as previously described (Sigler and Zeyer, 2002). PCR was performed in triplicate according to the protocol of Muyzer et al. (1993) with three different sets of oligonucleotide primers in order to generate three PCR product lengths for DGGE analysis. We used primers 341f-gc and 534r (~ 200 bp; Muyzer et al., 1993), 101f-gc and 537r (~ 440 bp; Schmalenberger

et al., 2001) and 101f-gc and 705r (~ 600 bp; Klijn et al., 1991) to generate a range of product lengths commonly used in DGGE. The total volume of product necessary to satisfy the requirements of the entire experiment was produced during a single PCR run, then aliquoted (25 µl) and frozen at -20 °C until DGGE analysis was performed. All reactions produced a single DNA band of the correct size as estimated by agarose gel electrophoresis and comparison with a DNA size standard (not shown).

For all DGGE experiments we poured (7.5 ml min⁻¹) 15 × 15 cm gels containing 8% acrylamide:bis-acrylamide (37.5:1) and 2% glycerol, with a denaturing gradient of formamide and urea of 25–65%. Because 1000 V·h represented a popular value among many published DGGE protocols, we chose it as a constant parameter to address the likelihood that varying the combination of voltage and time impacts the assessment of microbial community structure. Triplicate samples of each PCR product were subjected to DGGE under one of four combinations of voltage and time; 25 V for 40 h, 60 V for 16.7 h, 100 V for 10 h, and 200 V for 5 h (Fig. 1). For each combination, we monitored the resistance (mΩ) during the run, which did not change appreciably. For each triplicate DGGE lane, band number and position were assessed for pattern similarity using Quantity One image analysis software (Bio Rad Laboratories, Hercules CA) and three single-value indices of similarity were calculated; (1) phylotype richness (*S*, the number of bands); (2) Dice similarity (*S*_D),

$$S_D = \frac{2N_C}{N_Q + N_T},$$

where *N*_Q represented the number of bands detected in the query soil, *N*_T represented the number of bands detected in the test soil, and *N*_C represented the number of bands common to both soils; and (3) Jaccard similarity (*S*_J),

$$S_J = \frac{P_C}{P_T},$$

where *P*_T represented the total number of gel positions occupied by a band (i.e. the number of bands of different migration distance) and *P*_C represented the number of gel positions in which both lanes featured a band. Each lane's band richness was subjected to a

pairwise Student's *t*-test across the range of tested voltages in order to determine the significance of the differing electrophoresis regimes in assessing microbial community structure. Similarity indices were expressed numerically within a range of 0 (completely dissimilar) to 1.0 (perfect similarity). Results indicated that although the same V·h was applied during DGGE analysis, varying the electrophoresis time significantly altered band richness and impacted profile similarity (Table 1). Specifically, pairwise comparisons of band richness indicated significant differences in all evaluations with the exception of one pair (10 vs. 5 h, 400 bp). It is also interesting to note that for all fragment sizes, decreased electropho-

resis time resulted in a higher number of bands. It is unknown if a shorter run time would have resulted in a continued increase in band number, as 200 V represented the working limit of our electrophoresis setup, which limited our shortest run time to 5 h (while maintaining 1000 V·h). Profile similarity as assessed by both Dice- and Jaccard similarities indicated that altering the electrophoresis time promoted dissimilarity among all of the profiles. All profiles were found to be completely dissimilar to those resulting from DGGE at 25 V for 40 h (no bands in a common position). The highest similarities were observed upon comparing the 10- and 5-h regimes, but different fragment sizes produced the highest

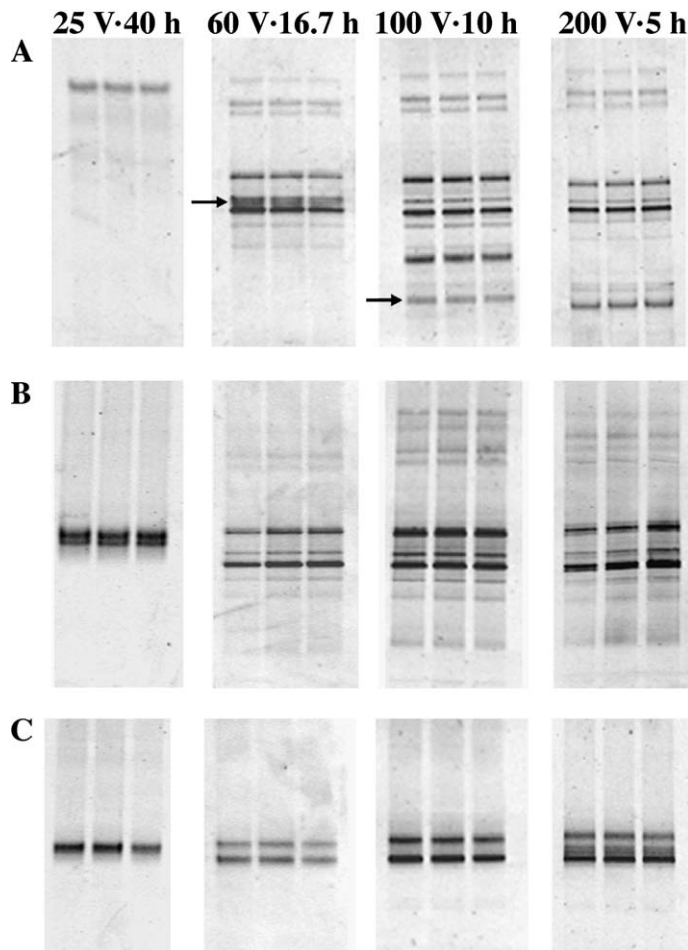


Fig. 1. DGGE analysis of (A) 200 bp-, (B) 440 bp-, and (C) 600 bp-PCR products under differing V·h. Previous experiments indicated the position of single-stranded DNA (arrows); these bands were not considered for the analyses in this study.

Table 1

Summary of DGGE band-based comparisons of band richness (S), and Dice- (S_D) and Jaccard (S_J) similarity indices

Index	Time (h)	200 bp				440 bp				600 bp			
		Electrophoresis time to achieve 1000 V·h (h)											
		40	16.7	10	5	40	16.7	10	5	40	16.7	10	5
Band richness (S)		1a ^a	8b	10c	11d	2.3a	7b	11.4c	13c	1a	2b	2c	3.4d
Dice similarity (S_D)	40		0	0	0		0	0	0		0	0	0
	16.7			0.33	0.11			0.36	0.35			0.50	0.40
	10				0.19				0.60				0.80
Jaccard similarity (S_J)	40		0	0	0		0	0	0		0	0	0
	16.7			0.20	0.06			0.21	0.21			0.33	0.25
	10				0.11				0.42				0.25

^a Richness values with different letters were significantly different as described in text.

similarities for the Dice- (600 bp; 0.80) and Jaccard (400 bp; 0.42) evaluations.

Based on our results, it is apparent that the selection of voltage–time ratio, not only the total V–H, has a significant impact on the ultimate band pattern generated during DGGE analysis as well as subsequent community structure assessments using all indices. By using the 200 bp product fingerprint as an example, we can conclude that in terms of estimated phylotype richness (S), which is a popular and easily quantified descriptor of DGGE results (Fromin et al., 2002), altering the electrophoresis time as described above would theoretically result in either a minimum of 1 phylotype- (25 V for 40 h) or a maximum of 11 phylotypes observed (200 V for 5 h) (Figs. 1A and 2A and B). Such discrepancy will have a significant effect on band richness and thus the overall community structure assessment. Although it could be argued that differences in the applied voltage, which ranged from 25 to 200 V, caused the dissimilarity in band patterns, this is probably not the case. It is more likely that instability of the denaturing gradient was the cause of the pattern differences, especially with longer electrophoresis times. To show this, we extracted DNA from five different bacterial isolates, performed PCR to obtain the 200 bp fragment, combined the PCR products, and then ran DGGE of the mixture for 5 h at 200 V. Two gels were prepared identically to those described above, however DGGE was performed either (i) 2 h after pouring the gel, or (ii) after incubating the gel for 35 h at 60 °C, which mimicked the 40 h electrophoresis time used in the first experiment. Although both samples were electro-

phoresed for 5 h at 200 V (1000 V·h), the pre-incubation at 60 °C for 35 h greatly impacted the separation and resolution of the isolate DNA (Fig. 2). This suggested that the prolonged incubation at an elevated temperature, and not the low voltage, played the major role in the differential band separation observed in Fig. 1. Although the exact reason for this separation phenomenon is unknown, it is possible that some internal diffusion of the denaturing gradient occurred following polymerization of the acrylamide. Furthermore, considering that the bottom of DGGE gels are open to the electrophoresis buffer, it is likely that diffusion of urea and formamide into the running buffer constantly occurs throughout the run, which promotes an enhanced breakdown of the gradient over longer runs.

Although DGGE fingerprinting has been recognized as an effective means for high throughput analysis of environmental samples (Ferrari and Holibaugh, 1999), the current study confirms that results of DGGE-based community structure analyses should be interpreted cautiously. Our results indicate that under a constant volt-hour regime, shorter electrophoresis times might minimize instability of the denaturing gradient and result in a more complete band separation than can be achieved following longer runs. Furthermore, it is recommended that gel-to-gel comparisons only be attempted if all gels have been subjected to similar electrophoresis parameters, as our results have illustrated that although the total electrophoresis V·h were consistent for each run, changing the electrophoresis time can greatly impact subsequent band-based analyses.

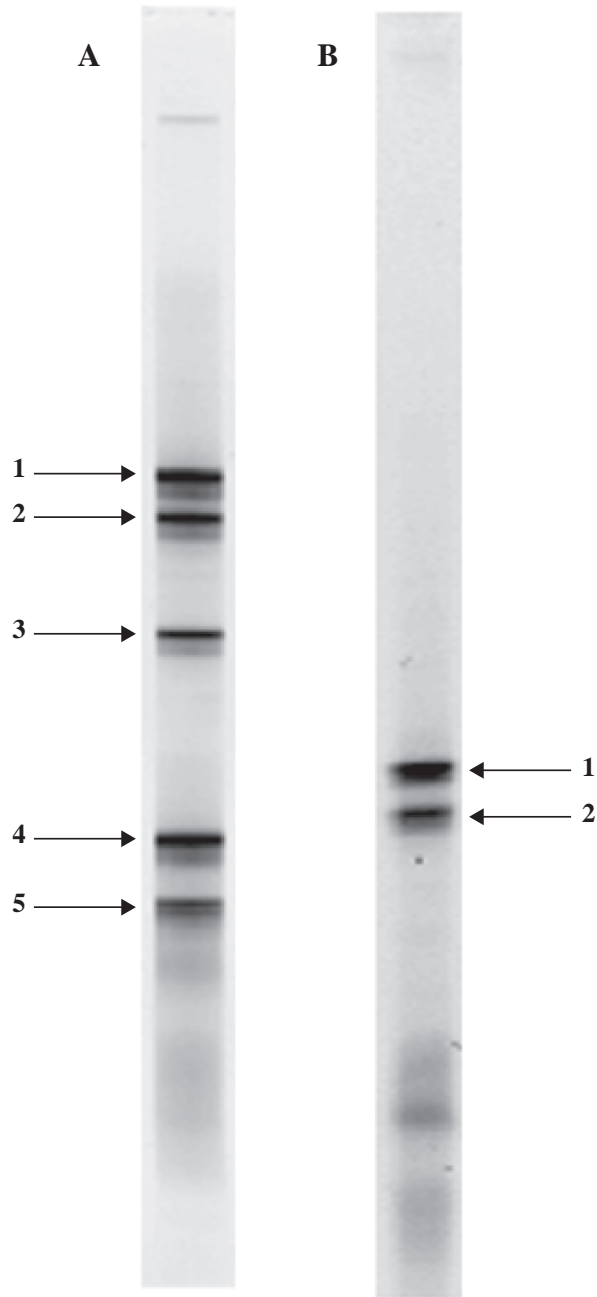


Fig. 2. DGGE of a five bacterial isolates (200 bp PCR product). DGGE was performed at 200 V for 5 h (A) 2 h after pouring the gel, or (B) following 35 h of pre-incubation at 60 °C. Isolates: (1) *Aeromonas baumannii*, (2) *Proteus mirabilis*, (3) *Ralstonia pickettii*, (4) *Escherichia coli*, (5) *Bacillus subtilis*.

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