Microbial Community Fingerprints by Differential Display Denaturing Gradient Gel Electrophoresis (DD-DGGE).

M.C. Portillo¹, D. Villahermosa², A. Corzo², J.M. Gonzalez¹*

¹ Instituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, Avda. Reina Mercedes 10, 41012-Sevilla, Spain.
² Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Polígono Río San Pedro s/n, 11510-Puerto Real, Spain.

* To whom correspondence should be addressed: Instituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, Avda. Reina Mercedes 10, 41012-Sevilla, Spain. Tel. +34 95 462 4711 (ext. 146) Fax +34 95 462 4002 E-mail: jmgrau@irnase.csic.es

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Abstract

Complex microbial communities exhibit a large diversity hampering differentiation by DNA fingerprinting. Herein, Differential Display Denaturing Gradient Gel Electrophoresis (DD-DGGE) is proposed. By adding a nucleotide to the 3’-end of PCR primers, 16 primer pairs and fingerprints were generated per community. Complexity reduction in each partial fingerprint facilitates sample comparison.
Microbial communities present a huge diversity which has been reported to exceed the possibilities of current molecular methods (4). This elevated diversity makes difficult the analysis of microbial communities and, above all, their comparison through time series, spatial distribution and other experimental treatments (16, 22).

Current analyses frequently involve the use of molecular fingerprinting methods as a relatively simple and rapid procedure to characterize each microbial community by a singular profile (7, 10, 18, 19). At present, different fingerprinting methods are available, for example, Denaturing Gradient Gel Electrophoresis (DGGE)(18), terminal-Restriction Fragment Length Polymorphisms (t-RFLP)(15), and Single Stranded Conformational Polymorphisms (SSCP)(21), among others. Most of the pitfalls usually attributed to fingerprinting techniques are inherent to PCR amplification (26, 27) and the potential bias generated during the protocol equally affects cloning and sequencing as well as any other PCR-based approaches (6, 14, 25) including new generation sequencing methods (12). Fingerprinting techniques are specially useful for the comparison of microbial communities and the detection of community shifts induced by different treatments (5, 16).

Shifts in microbial communities represent highly sensitive indicators of changes in a given system and this potential technology is of great interest in numerous scenarios (7). However, the large complexity of microbial communities often inhibits the detection of changes through fingerprinting analysis (1, 9). In this study, we propose a novel differential display fingerprinting method to discriminate DNA bands into different profiles. This discrimination is performed by the nucleotide following the 3’-end of the standard primers used in PCR amplification. Denaturing Gradient Gel
Electrophoresis (DGGE) is the fingerprinting technique used in this work, and the proposed differential display (DD-DGGE) of microbial community fingerprints is aimed to facilitate the detection of differences between microbial communities. This technique is applied to a case study differentiating control and nitrate-supplemented wastewaters.

**Materials and Methods.** Two reactors continuously operating with primary effluent from Guadalete Waste Water Treatment Plant (Jerez de la Frontera, Cadiz, Spain) were used in this study as previously described \(^9\). One of the reactors was supplemented with 0.24 mM calcium nitrate (final concentration; Nutriox\(^\text{TM}\)). Biofilms developed in the reactors were collected when maximum difference between the concentration of sulfide at control and supplemented reactors was observed \(^9\). Collected samples were stored frozen at -80°C until processed. DNA was extracted and 16S rRNA gene fragments were amplified by PCR following standard procedures described by Portillo et al. \(^{23}\) but using the primers described below (Table 1) and the PCR thermal conditions proposed for primer pair Bac8F and Bac356R \(^{13}\).

Microbial community fingerprints were carried out by DGGE \(^{18}\) using 6 h running time. The reverse primer Bac356R included a GC-rich tail sequence at its 5’-end for fragment stabilization during DGGE. Gels obtained by DGGE were digitalized and analyzed as previously described \(^{22}\) to estimate the quantitative ratios of selected bands in nitrate-supplemented versus control reactors.

Standard DGGE analyses resulted in molecular fingerprints with a high number of bands. In order to discriminate these bands into multiple molecular profiles, PCR
amplifications were performed with primer pairs designed by the standard priming sequence plus a different nucleotide at their 3’-end. Thus, a total of 16 primer pairs (A-A, A-C, A-G, A-T, C-A, C-C, etc.) were prepared and the amplified products of these 16 reactions were run into separate lanes. *In silico* evaluation of 16S rRNA gene sequences from RDP (Ribosomal Database Project)(3) showed that the sequences from different bacteria corresponded to distinct modified 3’-end primer pairs (Table 1) although a clear distribution of whole phyla within single primer pair combinations could not be established. Selected bands, showing different intensity in both treatments or absent in one of them, were cut-off the electrophoresis gels, reamplified and sequenced (29). Obtained sequences were submitted for homology searches (2) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Blast/) and deposited in GenBank under the accession numbers reported in Table 2.

Results and discussion. Differential display techniques has been long applied in the clinic environment preferentially focused on comparative analyses of gene expression (11, 20, 28). In this study we applied a similar principle using selective 3’-end primer pairs for PCR amplification combined with a fingerprinting technique (DGGE) resulting in the DD-DGGE procedure which greatly improves the potential to differentiate between bacterial communities and to detect specific bacterial phylotypes when comparing complex microbial systems.

The discrimination of amplicons into different PCR reactions based on a differential 3’-end nucleotide of the amplification primers resulted in a distribution of the total bacterial community fingerprints into several subcommunities characterized by a specific fingerprint. Some combinations of the forward and reverse primers used for
DD-DGGE (Table 1) did not select any amplicon and most members of the community were distributed into six combinations of modified 3’-end forward and reverse primers. This result is in agreement to *in silico* evaluation of the proposed protocol which confirmed that some primer combinations rarely correspond to reported bacterial 16s rRNA gene sequences from RDP. Figure 1 shows the subcommunity fingerprints obtained from each of the successful combinations of primer pairs. Great similarity between the total community fingerprints from the nitrate-supplemented and control reactors was observed and no differences in the banding pattern were clearly visualized (Figure 1T). This suggested that these communities presented scarce differences and that the DGGE procedure (i.e., PCR amplification, DGGE, and DD-DGGE) were highly reproducible. The subcommunity fingerprints (Figure 1, from A to F) showed a distribution of the total number of bands in several profiles and even allowed the detection of additional bands corresponding to bacterial 16S rRNA genes remaining undetected in the total community fingerprint. The subcommunity profiles showed lower complexity that the global fingerprint allowing the detection of several differences between control and nitrate-supplemented reactors.

Some of the most clear differences between the bacterial communities of nitrate-supplemented and control reactors are indicated in Figure 1 (labeled from ‘a’ to ‘q’). Table 2 shows the taxonomic affiliation of the bacteria corresponding to these selected bands. Besides, densitometric analysis of the DD-DGGE fingerprints allowed to quantify the proportion that each selected band represented in the nitrate-supplemented and control reactors (Figure 2). These bacteria showed an enhancement or inhibition during nitrate addition. Bacteria belonging to the Alphaproteobacteria (*Agrobacterium*), Firmicutes (within the Clostridiales), Betaproteobacteria (Burkholderiales), and
Actinobacteria (*Streptomyces*) were detected as being positively influenced by the addition of nitrate. However, some Alphaproteobacteria (Rhizobiales), Betaproteobacteria (Burkholderiales and Neisseriales), Firmicutes (different phylotypes within the Clostridiales), Acidobacteria, and Synergistetes were detected as being partially repressed by the supplemented nitrate.

The bacteria identified during this study do not correspond to major nitrate-reducing, sulfide-oxidizing bacteria as expected from previous studies (8). We have mainly detected heterotrophic bacteria generally reported in wastewaters but with scarce potential to decipher critical nutrient cycling steps other than organic carbon decomposition. This result suggests that PCR amplification combined with DGGE analysis (DD-DGGE in this study) is only able to detect the most abundant components of bacterial communities (above 1% of total community) (17, 18). In an scenario dominated by heterotrophic bacteria, those representing a minor portion of the studied communities would only be occasionally detected using domain-wide primer pairs. The proportion of *Sulfurimonas*-like relatives detected using group-specific primers by Garcia-de-Lomas et al. (8) constituted at most a 0.1% of the total bacterial community. These results are in agreement with the high bacterial diversity reported in wastewaters (1, 9, 24) and indicates that DD-DGGE is a useful method to comparatively analyze the abundant members of bacterial communities from different treatments or sampling series. In the present work, the discrimination of bacterial phylotypes into different PCR amplification reactions and their display by DD-DGGE allowed to simplify the DNA banding profiles and also improved the number of detected bands when compared to standard PCR and DGGE procedures.
Herein, a novel strategy for the comparative analysis of bacterial communities is proposed by combining 3’-end discriminating primer pairs for PCR amplification and DGGE fingerprinting to obtain multiple subcommunities. The proposed procedure, DD-DGGE, provides with a platform for the simple and rapid comparison of bacterial communities and the detection of their major components showing differential behavior between samples.

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References


amplicons for rapid deciphering of the microbiomes of fermented foods such as

R. McDermott. 2001. Molecular analysis of microbial community structure in an

abundance of an uncultured soil bacterial strain by a competitive quantitative PCR

microbial diversity by determining terminal restriction fragment length
polymorphisms of genes encoding 16s rRNA. Appl. Environ. Microbiol. 63:4516-
4522.

grassland bacterial community structure under different land management regimens
by using 16s ribosomal DNA sequence data and denaturing gradient gel

compositions of bacterioplankton from two California estuaries compared by
denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl. Environ.

microbial populations by denaturing gradient gel electrophoresis analysis of
polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ.
Microbiol. 59:695-700.


Figure legends

Figure 1. Molecular fingerprint of the total bacterial community (T) and partial community (A-F) of samples from reactors with control (Co) and nitrate-supplemented (Ni) wastewater. Partial community fingerprints correspond to those obtained by using different 3’-ending priming sequences (Table 1): A, A (forward, Bac8Fa) and A (reverse, Bac356Ra); B, A (forward, Bac8Fa) and G (reverse, Bac356Rg); C, C (forward, Bac8Fc) and G (reverse, Bac356Rg); D, G (forward, Bac8Fg) and A (reverse, Bac356Ra); E, G (forward, Bac8Fg) and T (reverse, Bac356Rt); F, G (forward, Bac8Fg) and G (reverse, Bac356Rg); T, total community (forward, Bac8F; reverse Bac356R). The identified OTUs are labelled as in Table 2.

Figure 2. Quantitative analysis of fluorescence intensity for the community fingerprints from control (red lines) and nitrate-supplemented (blue lines) reactors compared in Figure 1. The peaks corresponding to identified OTUs are labelled as in Table 2.
Table 1. Oligonucleotides used for PCR amplification in this study. Primers used for DD-DGGE are the primers used for total community amplification plus a single nucleotide (in bold) added at the 3’-end.

<table>
<thead>
<tr>
<th>ID</th>
<th>Oligonucleotide sequence (5’→3’)</th>
<th>Reference</th>
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<tbody>
<tr>
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<td><strong>DGGE oligonucleotides</strong></td>
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<tr>
<td>Bac8F</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
<td>Jackson et al. 2001</td>
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<td>Bac356R</td>
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</tr>
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<td></td>
<td><strong>DD-DGGE oligonucleotides</strong></td>
<td></td>
</tr>
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<td>Bac8Fa</td>
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</tr>
<tr>
<td>Bac8Fc</td>
<td>AGAGTTTGATCCTGGCTCAG**C</td>
<td>This study</td>
</tr>
<tr>
<td>Bac8Fg</td>
<td>AGAGTTTGATCCTGGCTCAG**G</td>
<td>This study</td>
</tr>
<tr>
<td>Bac8Ft</td>
<td>AGAGTTTGATCCTGGCTCAG**T</td>
<td>This study</td>
</tr>
<tr>
<td>Bac356Ra</td>
<td>*GCTGCCTCCCGTAGGAGT**A</td>
<td>This study</td>
</tr>
<tr>
<td>Bac356Rc</td>
<td>*GCTGCCTCCCGTAGGAGT**C</td>
<td>This study</td>
</tr>
<tr>
<td>Bac356Rg</td>
<td>*GCTGCCTCCCGTAGGAGT**G</td>
<td>This study</td>
</tr>
<tr>
<td>Bac356Rt</td>
<td>*GCTGCCTCCCGTAGGAGT**T</td>
<td>This study</td>
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* A GC-rich tail (5’-CGCCCGCCCGCCGCGCGGCGGGGGGCGGGGCACGGGGG) was incorporated to the 5’-end of the reverse primers.
Table 2. List of bands selected during DD-DGGE analysis and their taxonomic classification. The ratio of nitrate-supplemented reactor vs. control reactor (ratio Ni/Co) from densitometric analysis of fingerprints is shown. Ratios below one indicate higher values in the control reactor while ratios above one suggest an enhancement due to the supplemented nitrate. OTUs are labelled as in Figure 2.

<table>
<thead>
<tr>
<th>OTU</th>
<th>Ratio Ni/Co</th>
<th>Accession number</th>
<th>Taxonomic classification</th>
<th>Accession number of closest homologue</th>
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<td>a</td>
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<td><em>Comamonas</em>, Betaproteobacteria</td>
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<td>-</td>
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<td>HM440959</td>
<td>Acidobacteria</td>
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<td>Clostridiales, Firmicutes</td>
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<td><em>Acidovorax</em>, Betaproteobacteria</td>
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<td>EF188662</td>
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<tr>
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<td>GU640852</td>
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<td><em>Streptomyces</em>, Actinobacteria</td>
<td>GU550566</td>
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<td>CU925891</td>
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<tr>
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<td>Synergistetes</td>
<td>CU924713</td>
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<td>q</td>
<td>0.340</td>
<td>HM440964</td>
<td>Synergistetes</td>
<td>EU837979</td>
</tr>
</tbody>
</table>
Figure 2

A

B

C

D

E

F

X (pixels)

Fluorescence intensity

0 100 200 300 400 500 600 700 800

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 2

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

0 100 200 300 400 500 600 700 800

X (pixels)

Fluorescence intensity

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0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 2

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Fluorescence intensity

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Fluorescence intensity

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0 100 200 300 400 500 600 700 800

X (pixels)

Fluorescence intensity

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0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1 2

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

0 100 200 300 400 500 600 700 800

X (pixels)

Fluorescence intensity

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8

0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1

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0 100 200 300 400 500 600 700 800

X (pixels)

Fluorescence intensity