Microbial Community Fingerprinting by Differential Display-Denaturing Gradient Gel Electrophoresis

M. C. Portillo, D. Villahermosa, A. Corzo and J. M. Gonzalez
Appl. Environ. Microbiol. **2011, 77(1):351. DOI: 10.1128/AEM.01316-10.**
Published Ahead of Print 12 November 2010.

Applied and Environmental Microbiology, Jan. 2011, p. 351–354 Vol. 77, No. 1
0099-2240/11/$12.00 doi:10.1128/AEM.01316-10

Microbial Community Fingerprinting by Differential Display-Denaturing Gradient Gel Electrophoresis▽

M. C. Portillo,[1] D. Villahermosa,[2] A. Corzo,[2] and J. M. Gonzalez[1]*

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

Received 3 June 2010/Accepted 1 November 2010

Complex microbial communities exhibit a large diversity, hampering differentiation by DNA fingerprinting. Herein, differential display-denaturing gradient gel electrophoresis is proposed. By adding a nucleotide to the 3′ ends 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 abilities 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 single profile (7, 10, 18, 19). At present, different fingerprinting methods are available, for example, denaturing gradient gel electrophoresis (DGGE) (18), terminal restriction fragment length polymorphism (15), and single-stranded conformational polymorphism (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 especially 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 (DD) 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. DGGE is the fingerprinting technique used in this work, and the proposed 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.

* Corresponding author. Mailing address: Instituto de Recursos Naturales y Agrobiología, IRNAS-CSIC, Avda. Reina Mercedes 10, 41012-Seville, Spain. Phone: 34 95 462 4711, ext. 146. Fax: 34 95 462 4002. E-mail: jmgrau@irnase.csic.es.

▽ Published ahead of print on 12 November 2010.

Materials and methods. Two reactors continuously operating with primary effluent from the Guadalete wastewater 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 (final concentration) calcium nitrate (Nutriox). Biofilms developed in the reactors were collected when a maximum difference between the sulfide concentrations in the 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 primers Bac8F and Bac356R (13).

Microbial community fingerprints were carried out by DGGE (18) using a 6-h running time. Reverse primer Bac356R included a GC-rich tail sequence at its 5′ end for fragment stabilization during DGGE. Gels obtained by DGGE were digitized 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

TABLE 1. Oligonucleotides used for PCR amplification in this study[a]

Oligonucleotide	Sequence (5′–3′)	Reference
DGGE		
Bac8F	AGAGTTTGATCCTGGCTCAG	13
Bac356R	GCTGCCTCCCGTAGGAGT[b]	13
DD-DGGE		
Bac8Fa	AGAGTTTGATCCTGGCTCAG**A**	This study
Bac8Fc	AGAGTTTGATCCTGGCTCAG**C**	This study
Bac8Fg	AGAGTTTGATCCTGGCTCAG**G**	This study
Bac8Ft	AGAGTTTGATCCTGGCTCAG**T**	This study
Bac356Ra	GCTGCCTCCCGTAGGAGT**A**[b]	This study
Bac356Rc	GCTGCCTCCCGTAGGAGT**C**[b]	This study
Bac356Rg	GCTGCCTCCCGTAGGAGT**G**[b]	This study
Bac356Rt	GCTGCCTCCCGTAGGAGT**T**[b]	This study

[a] The primers used for DD-DGGE are the primers used for total-community amplification plus a single nucleotide (in bold) added at the 3′ end.
[b] A GC-rich tail (5′-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGC ACGGGGGG) was incorporated at the 5′ end of this reverse primer.

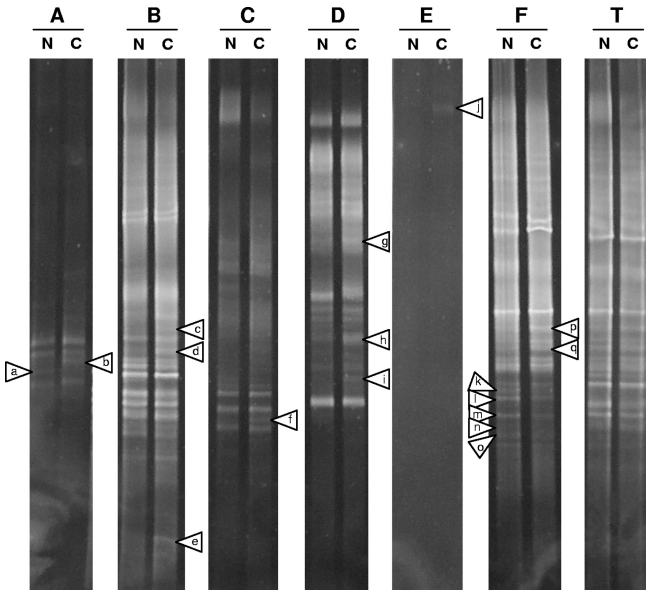

FIG. 1. Molecular fingerprints of the total bacterial community (T) and partial communities (A to F) of samples from reactors with control (C) and nitrate-supplemented (N) wastewater. Partial community fingerprints correspond to those obtained by using different 3′-end primer sequences (Table 1) as follows: 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 operational taxonomic units identified are labeled as in Table 2.

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 the 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 in 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 intensities in the two treatments or absent from one of them were cut off the electrophoresis gels, reamplified, and sequenced (28). The sequences obtained were submitted for homology searches (2) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Blast/).

Results and discussion. DD techniques have long been applied in the clinic environment, preferentially focused on comparative analyses of gene expression (11, 20, 29). 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 ability to differentiate between bacterial communities and detect specific bacterial phylotypes when comparing complex microbial systems.

The discrimination of amplicons into different PCRs 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 specific fingerprints. Some combinations of the forward and reverse primers used for DD-DGGE (Table 1) did not select any amplicon, and most of the members of the community were distributed into six combinations of modified 3′-end forward and reverse primers. This result is in agreement with an *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 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 patterns were clearly visualized (Fig. 1T). This suggested that these communities presented scarce differences and that the DGGE procedure (i.e., PCR amplification,

TABLE 2. Bands selected during DD-DGGE analysis and their taxonomic classifications[a]

OTU	Ni/Co ratio	Accession no.	Taxonomic classification	Accession no. of closest homologue
a	2.504	HM440961	*Agrobacterium*, *Alphaproteobacteria*	GU569146
b	0.250	HM440962	*Rhizobiales*, *Alphaproteobacteria*	FN436205
c	0.566	HM440957	*Burkholderiales*, *Betaproteobacteria*	FN550733
d	0.333	HM440963	*Neisseriales*, *Betaproteobacteria*	CP001154
e	0.401	HM440960	*Comamonas*, *Betaproteobacteria*	CU926399
f	0.393		Unidentified	
g	0.368	HM440972	*Clostridiales*, *Firmicutes*	CU926233
h	2.496	HM440958	*Clostridiales*, *Firmicutes*	GU303775
i	0.276	HM440959	*Acidobacteria*	CU927180
j	0.001	HM440971	*Clostridiales*, *Firmicutes*	CU925306
k	2.269	HM440970	*Acidovorax*, *Betaproteobacteria*	FN794211
l	1.510	HM440969	*Rhizobiales*, *Alphaproteobacteria*	EF188662
m	1.183	HM440968	*Burkholderiales*, *Betaproteobacteria*	GU640852
n	1.648	HM440967	*Streptomyces*, *Actinobacteria*	GU550566
o	2.846	HM440966	*Clostridiales*, *Firmicutes*	CU925891
p	0.413	HM440965	*Synergistetes*	CU924713
q	0.340	HM440964	*Synergistetes*	EU837979

[a] The ratio of the nitrate-supplemented reactor to the control reactor (Ni/Co) from a densitometric analysis of fingerprints is shown. Ratios below 1 indicate higher values in the control reactor, while ratios above 1 suggest enhancement due to nitrate supplementation. Operational taxonomic units (OTUs) are labeled as in Fig. 2.

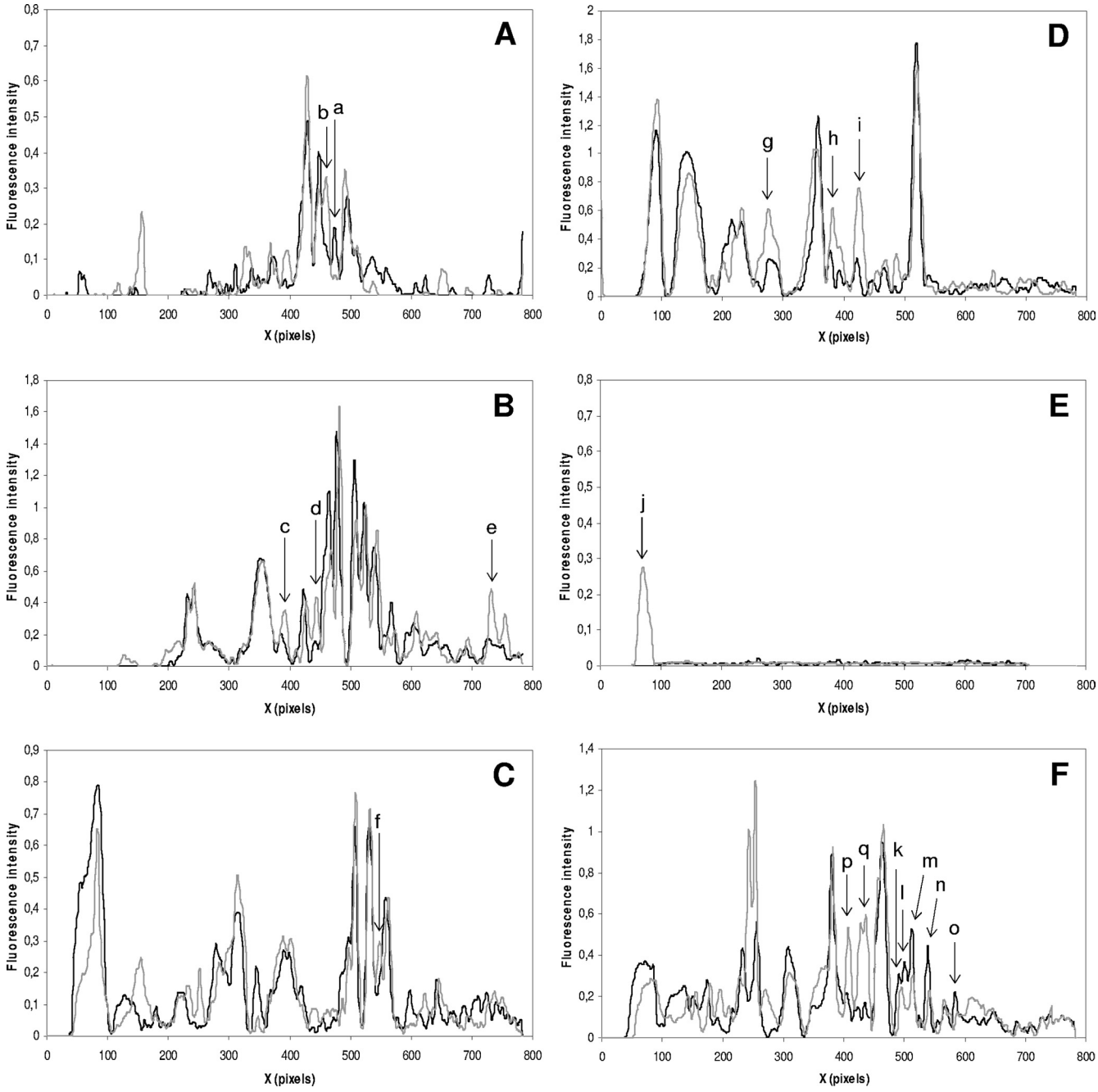

FIG. 2. Quantitative analysis of the fluorescence intensities of the community fingerprints from the control (grey lines) and nitrate-supplemented (black lines) reactors compared in Fig. 1. The peaks corresponding to the operational taxonomic units identified are labeled as in Table 2.

DGGE, and DD-DGGE) were highly reproducible. The subcommunity fingerprints (Fig. 1A 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 fingerprints. The subcommunity profiles showed less complexity that the global fingerprints, allowing the detection of several differences between the control and nitrate-supplemented reactors.

Some of the clearest differences between the bacterial communities of our nitrate-supplemented and control reactors are indicated in Fig. 1 (labeled 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 us to quantify the proportion that each selected band represented in the nitrate-supplemented and control reactors (Fig. 2). These bacteria showed 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 nitrate supplementation.

The bacteria identified during this study do not correspond to major nitrate-reducing, sulfide-oxidizing bacteria, as expected from a previous study (8). We have detected mainly heterotrophic bacteria generally reported in wastewaters but with scarce potential to decipher critical nutrient-cycling steps other that organic carbon decomposition. This result suggests that PCR amplification combined with DGGE analysis (DD-DGGE in this study) is able to detect only the most abundant components of bacterial communities (>1% of the total community) (17, 18). In a scenario dominated by heterotrophic bacteria, those representing a minor portion of the communities studied would only be occasionally detected using domain-wide primer pairs. The proportion of *Sulfurimonas*-like relatives detected using group-specific primers by García de Lomas et al. (8) constituted, at most, 0.1% of the total bacterial community. These results are in agreement with the high bacterial diversity reported in wastewaters (1, 9, 24) and indicate 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 amplifications and their display by DD-DGGE allowed us to simplify the DNA banding profiles and also improved the number of bands detected compared to the number detected by 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 a platform for the simple and rapid comparison of bacterial communities and the detection of their major components showing differential behavior between samples.

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study were deposited in GenBank under the accession numbers reported in Table 2.

This work was supported mainly by grant P06-RNM-01787 from the Consejería de Innovación, Ciencia y Empresa, Junta de Andalucía, Spain, and by projects CGL2009-12328/BOS and Consolider CSD2009-00006 from the Spanish Ministry of Science and Innovation.

We acknowledge Aguas de Jerez E.M.S.A. for the use of the Guadalete wastewater treatment plant and for technical assistance and E. Iglesias from Yara Iberian for providing Nutriox.

REFERENCES

1. **Akarsubasi, A. T., O. Eyice, I. Miskin, I. M. Head, and T. P. Curtis.** 2009. Effect of sludge age on the bacterial diversity of bench scale sequencing batch reactors. Environ. Sci. Technol. **43:**2950–2956.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
3. **Cole, J. R., B. Chai, R. J. Farris, Q. Wang, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, A. M. Bandela, E. Cardenas, G. M. Garrity, and J. M. Tiedje.** 2007. The Ribosomal Database Project (RDP-II): introducing *myRDP* space and quality controlled public data. Nucleic Acids Res. **35:** D169–D172.
4. **Curtis, T. P., W. T. Sloan, and J. W. Scannell.** 2002. Estimating prokaryotic diversity and its limits. Proc. Natl. Acad. Sci. U. S. A. **99:**10494–10499.
5. **Duineveld, B. M., A. S. Rosado, J. D. van Elsas, and J. A. van Veen.** 1998. Analysis of the dynamics of bacterial communities in the rhizosphere of the *Chrysanthemum* via denaturing gradient gel electrophoresis and substrate utilization patterns. Appl. Environ. Microbiol. **64:**4950–4957.
6. **Fisher, M. M., and E. W. Triplett.** 1999. Automated approach for ribosomal intergenic spacer analysis of microbial diversity and its application to freshwater bacterial communities. Appl. Environ. Microbiol. **65:**4630–4636.
7. **Fromin, N., J. Hamelin, S. Tarnawski, D. Roesti, K. Jourdain-Miserez, N. Forestier, S. Teyssier-Cuvelle, F. Gillet, M. Aragno, and P. Rossi.** 2002. Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ. Microbiol. **4:**634–643.
8. **García de Lomas, J., A. Corzo, J. M. Gonzalez, J. A. Andrades, E. Iglesias, and M. J. Montero.** 2006. Nitrate promotes biological oxidation of sulfide in wastewaters: experiment at plant-scale. Bioeng. Biotechnol. **93:**801–811.
9. **Garcia-de-Lomas, J., A. Corzo, M. C. Portillo, J. M. Gonzalez, J. A. Andrades, and C. Saiz-Jimenez.** 2007. Nitrate stimulation of indigenous nitrate-reducing, sulfide-oxidising bacterial community in wastewater anaerobic biofilms. Water Res. **41:**3121–3131.
10. **Gonzalez, J. M., and C. Saiz-Jimenez.** 2004. Microbial diversity in biodeteriorated monuments as studied by denaturing gradient gel electrophoresis. J. Sep. Sci. **27:**174–180.
11. **Guimarães, M. J., F. Lee, A. Zlotnik, and T. McClanahan.** 1995. Differential display by PCR: novel findings and applications. Nucleic Acids Res. **23:**1832–1833.
12. **Humblot, C., and J.-P. Guyot.** 2009. Pyrosequencing of tagged 16S rRNA gene amplicons for rapid deciphering of the microbiomes of fermented foods such as pearl millet slurries. Appl. Environ. Microbiol. **75:**4354–4361.
13. **Jackson, C. R., H. W. Langer, J. Donahoe-Christiansen, W. P. Inskeep, and T. R. McDermott.** 2001. Molecular analysis of microbial community structure in an arsenite-oxidizing acidic thermal spring. Environ. Microbiol. **3:**532–542.
14. **Lee, S. Y., J. Bollinger, D. Bezdicek, and A. Ogram.** 1996. Estimation of the abundance of an uncultured soil bacterial strain by a competitive quantitative PCR method. Appl. Environ. Microbiol. **62:**3787–3793.
15. **Liu, W. T., T. L. Martin, H. Cheng, and L. J. Formey.** 1997. Characterization of microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Appl. Environ. Microbiol. **63:**4516–4522.
16. **McCaig, A. E., L. A. Glover, and J. I. Prosser.** 2001. Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbiol. **67:**4554–4559.
17. **Murray, A. E., J. T. Hollibaugh, and C. Orrego.** 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. Appl. Environ. Microbiol. **62:**2676–2680.
18. **Muyzer, G., E. C. de Waal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. **59:**695–700.
19. **Muyzer, G., and K. Smalla.** 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie Van Leeuwenhoek **73:**127–141.
20. **Pavon, L. F., M. I. C. Mathias, F. Meirelles, and M. B. Júnior.** 2006. Analysis of the differential gene expression, by the DD-RTPCR technique, of workers *Atta sexdens rubropilosa* mandibular glands (Hymenoptera: Formicidae). Am. J. Agric. Biol. Sci. **1:**36–43.
21. **Peu, P., H. Brugere, A. M. Pourcher, M. Kerouredan, J. J. Godon, J. P. Delgenes, and P. Dabert.** 2006. Dynamics of a pig slurry microbial community during anaerobic storage and management. Appl. Environ. Microbiol. **72:**3578–3585.
22. **Portillo, M. C., and J. M. Gonzalez.** 2008. Statistical differences between molecular fingerprints from microbial communities. Antonie Van Leeuwenhoek **94:**157–163.
23. **Portillo, M. C., V. Sririn, W. Kanoksilapatham, and J. M. Gonzalez.** 2009. Different microbial communities in hot spring mats from western Thailand. Extremophiles **13:**321–331.
24. **Rowan, A. K., J. R. Snape, D. Fearnside, M. R. Barer, T. P. Curtis, and I. M. Head.** 2003. Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different design treating identical wastewater. FEMS Microbiol. Ecol. **43:**195–206.
25. **Schäfer, H., and G. Muyzer.** 2001. Denaturing gradient gel electrophoresis in marine microbial ecology. Methods Microbiol. **30:**425–468.
26. **Suzuki, M., and S. Giovanonni.** 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Appl. Environ. Microbiol. **62:**625–630.
27. **von Wintzingerode, F., U. B. Göbel, and E. Stackebrandt.** 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. **21:**213–229.
28. **Xia, S., Y. Shi, Y. Fu, and X. Ma.** 2005. DGGE analysis of 16S rDNA of ammonia-oxidizing bacteria in chemical-biological flocculation and chemical coagulation systems. Appl. Microbiol. Biotechnol. **69:**99–105.
29. **Yang, S., and P. Liang.** 2004. Global analysis of gene expression by differential display. Mol. Biotechnol. **27:**197–208.