Microbial phyllosphere populations are more complex than previously realized

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Phyllosphere microbial communities were evaluated on leaves of field-grown plant species by culture-dependent and -independent methods. Denaturing gradient gel electrophoresis (DGGE) with 16S rDNA primers generally indicated that microbial community structures were similar on different individuals of the same plant species, but unique on different plant species. Phyllosphere bacteria were identified from *Citrus sinesis* (cv. Valencia) by using DGGE analysis followed by cloning and sequencing of the dominant rDNA bands. Of the 17 unique sequences obtained, database queries showed only four strains that had been described previously as phyllosphere bacteria. Five of the 17 sequences had 16S similarities lower than 90% to database entries, suggesting that they represent previously undescribed species. In addition, three fungal species were also identified. Very different 16S rDNA DGGE banding profiles were obtained when replicate cv. Valencia leaf samples were cultured in BIOLOG EcoPlates for 4.5 days. All of these rDNA sequences had 97–100% similarity to those of known phyllosphere bacteria, but only two of them matched those identified by the culture independent DGGE analysis. Like other studied ecosystems, microbial phyllosphere communities therefore are more complex than previously thought, based on conventional culture-based methods.

All plant species in natural habitats have associated epiphytic microflora comprising the so-called phyllosphere (1, 2). The composition and quantity of nutrients, including carbohydrates, organic acids, and amino acids that support the growth of epiphytic bacteria, are affected by the plant species, leaf age, leaf physiological status, and the presence of tissue damage (3). Similarly, host plants, leaf age, leaf position, physical environmental condition, and availability of immigrant inoculum have also been suggested to be involved in determining species of microbes in the phyllosphere (4–7).

There has been much interest in life forms that inhabit extreme environments such as the phyllosphere. With the repeated, rapid alteration of environmental conditions occurring on leaf surfaces, the phyllosphere has been recognized as a hostile environment to bacteria (8). Leaf surfaces are often dry and temperatures can reach 40–55°C under intense sunlight. During the night, however, leaves are frequently wet with dew and at relatively cool temperatures (5–10°C). Strong UV radiation during the day and sparse nutritional (oligotrophic) conditions also contribute to stressful conditions in the phyllosphere (8). More than 85 different species of microorganisms in 37 genera have been reported in the phyllospheres of rye, olive, sugar beet, and wheat, all by culture-based methods (8–10). Most of these bacteria establish large populations with no apparent effect on the plant, but a few of them can infect the leaves and cause disease (1).

Microbial ecologists have devoted much effort to investigating microbial diversity and studying biological interactions between species in the environment. Microorganisms are typically plated on laboratory culture media, and different types of colonies are identified with biochemical or morphological methods (11, 12). Microbial communities can also be analyzed with carbon-source utilization patterns through the application of environmental samples into BIOLOG microplates (13, 14). Other methods, including fatty acid methyl ester (FAME), phospholipid fatty acid ester (PLFA), $C_0t_{1/2}$ curve analysis, and denaturing gradient gel electrophoresis (DGGE) have also been applied to analyze microbial communities with or without culturing microbes (15–19). In virtually all cases, culture-independent methods have revealed more complexity in the microbial populations of particular ecosystems than culture-based methods. Although culture-independent methods have been widely used on samples from soil, water, and the rhizosphere, they have not been used to examine the phyllosphere (8, 12, 20–26).

In this study, phyllosphere communities on seven different plant species were evaluated with culture-dependent and -independent methods. Strikingly, culture-independent methods revealed that most dominant phyllosphere organisms were not detected by conventional culture-based methods.

Materials and Methods

Leaf Sampling. Leaf samples of OroBlanco [*Citrus maxima* (Burm.) Merrill × *C. paradisi* Macf. (grapefruit hybrid)], Valencia orange [*C. sinensis* (L.) Osbeck], navel orange (*C. sinensis*), cotton [*Gossypium hirsutum* L. (Deltapine DP5415)], corn [*Zea mays* L. (inbred B73, Pioneer Hi-brid International)], sugar beet [*Beta vulgaris* L. var. *Saccharifera* (Spraekels SS-NB)], and green bean [*Phaseolus vulgaris* L. (Blue Lake 274)] were collected from the Agricultural Experiment Station at the University of California, Riverside. Cotton, corn, sugar beets, and green beans were grown adjacent to each other in field no. 10, plots F, G, H, and I, with plot sizes measuring 51 × 55 m, respectively, for each crop. Valencia orange and grapefruit plants were grown in plot L (field no. 16), which measured 70 × 104 m. Navel orange plants were grown in field no. 16, plot K. Plot K, with a size similar to plot L, was next to plot L. Field nos. 10 and 16 were separated by a 1.1-km linear distance. For sampling leaves, fully expanded young leaves of each plant were collected and placed on ice. Cotton and corn leaves were collected before the fruiting stage. Green beans were collected at the predehiscent stage. Grapefruit, and Valencia and navel orange trees were 8, 31, and 15 years old, respectively. Leaf samples were collected on October 9, 2000 and brought back to the laboratory at 4°C, and microbes were directly extracted from the leaf surface. Three individual plants were sampled from each plant species. We took 20 g of leaves from each plant, and three plants were sampled for each species/cultivar.

Extraction of DNA from Phyllosphere Microorganisms. Leaves were placed in polypropylene tubes and submerged with washing buffer (0.1 M potassium phosphate buffer, pH 7.0) and sonicated for 7 min in an ultrasonic cleaning bath (Bransonic 32) for 7 min to dislodge bacteria from leaves (5). Microbes were centrifuged

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Abbreviation: DGGE, denaturing gradient gel electrophoresis.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF231471–AF231497).

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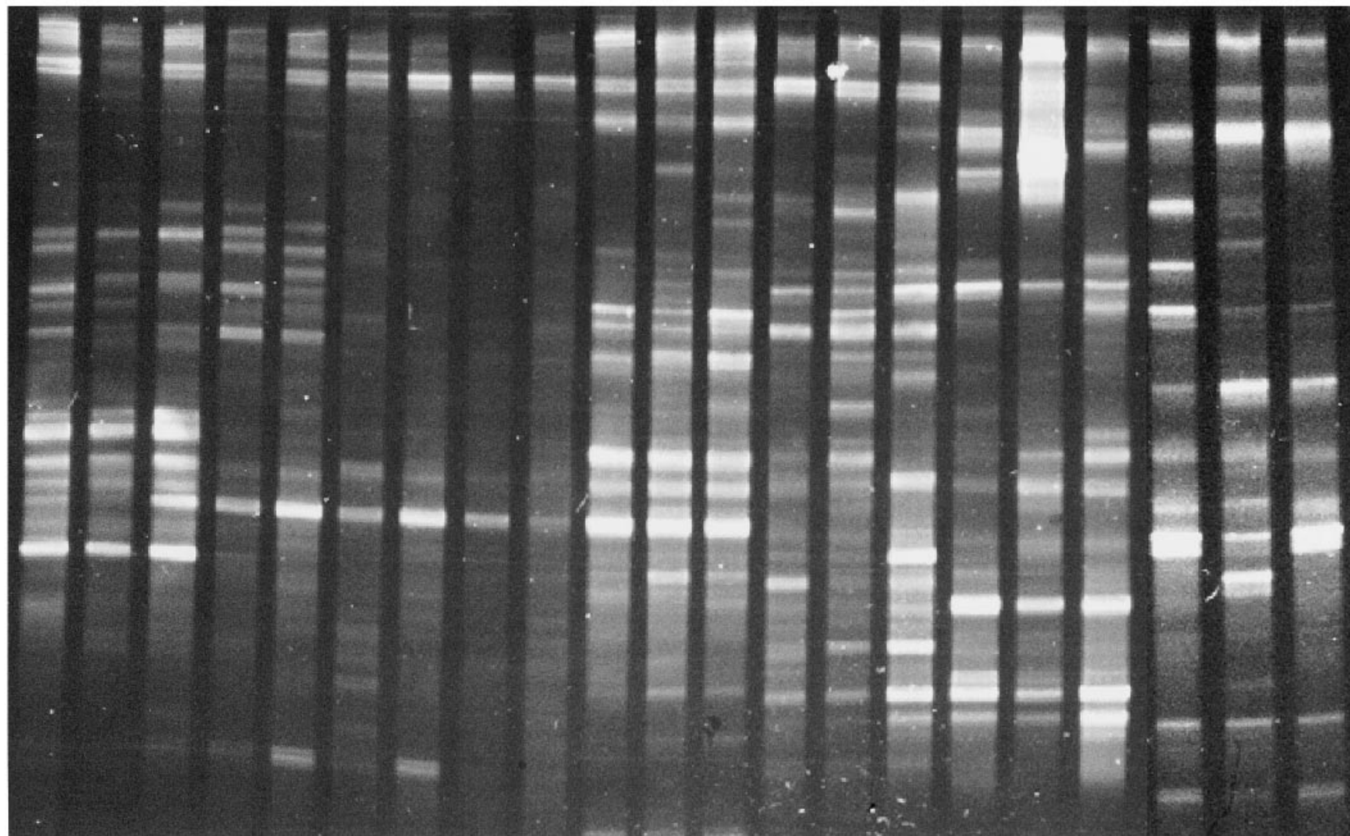

Fig. 1. PCR-DGGE 16S rDNA banding profiles of microorganisms from the phyllosphere of nine different plant crops. Lanes: 1–3, OroBlanco; 4–6, Valencia orange; 7–9, navel orange; 10–12, cotton; 13–15, corn; 16–18, sugar beet; 19–21, green bean.

at 30,000 g for 15 min, resuspended in phosphate buffer, and disrupted in tubes containing ceramic spheres (FastDNA kit, Bio 101) by a Fastprep FP120 Instrument (Bio 101) at a speed of 5.0 m/s for 30 s. Total DNA from the phyllosphere microorganisms was isolated with a FastDNA kit following the manufacturer's description.

Characterization of Bacterial Communities in BIOLOG EcoPlates by DGGE. Fifteen grams (about 20 leaves) of citrus Valencia orange leaves from three individual adjacent trees were collected on October 7, 1999. Phyllosphere bacteria were extracted as described above. Forty milliliters of leaf wash were centrifuged at $30{,}000 \times g$ for 15 min and resuspended in 6 ml of the phosphate buffer. Each bacterial suspension was inoculated into BIOLOG EcoPlates (150 μl into each well). One milliliter of the microbial suspension from each leaf sample was also used to extract the total DNA for microbe community assay by using DGGE. DNA was extracted with a FastDNA kit (Bio 101).

PCR Amplification. A 236-bp DNA fragment in the V3 region of the small subunit ribosomal RNA gene from the bacterioplankton community was amplified by using primer set PRBA338f and PRUN518r (27). For the purpose of separating these 16S rDNA bacterial communities in a DGGE gel, a GC clamp was added at the end of primer PRBA338f. PCR beads from Amersham Pharmacia Biotech were used to perform PCR. Five pmol of primers and a total of 25 μl of PCR mix were used. PCR amplifications were done with the following conditions: 92°C for 2 min; 30 cycles of 92°C for 1 min, 55°C for 30 s, 72°C for 1 min; and a single final extension for 6 min as described (27).

DGGE Analysis. DGGE was performed with a Dcode Universal Mutation Detection System (Bio-Rad). Twenty microliters of the PCR products from phyllosphere samples was loaded onto an 8% (wt/vol) acrylamide gel (acrylamide/bis solution, 37.5:1; Bio-Rad) containing a linear chemical gradient ranging from 20% to 70% denaturant [7 M urea and 40% (vol/vol) formamide]. The gels were run for 3 h at 200 V in 1× TAE electrophoresis buffer (0.04 M Tris-acetate/1 mM EDTA, pH 8.5). After electrophoresis, the gels were soaked in ethidium bromide solution for 10 min, rinsed for 5 min in water, and photographed with a Fluor-S MultiImager (Bio-Rad). The gel images were further transformed as digital data using Quantity One from Bio-Rad.

Statistical Analysis. The digitized gel images were straightened and aligned by using Adobe PHOTOSHOP 5.0 (Adobe Systems, San Jose, CA) and imported into an image analysis program (Scion Image, Scion Corp, Frederick, MD). The banding profiles from DGGE gels were converted into *x*/*y* plots, and transferred to EXCEL files (Microsoft). The peak area and distance (R_f) of each small subunit rDNA band were further analyzed using Peak Fit vs. 4 (SPSS, Chicago). Based on peak areas, the community similarities were further analyzed by cluster analysis (Minitab, State College, PA). Peaks from each DGGE band represented different bacterial species or groups of species. Similarities in community structure quantified by cluster analysis were deter-

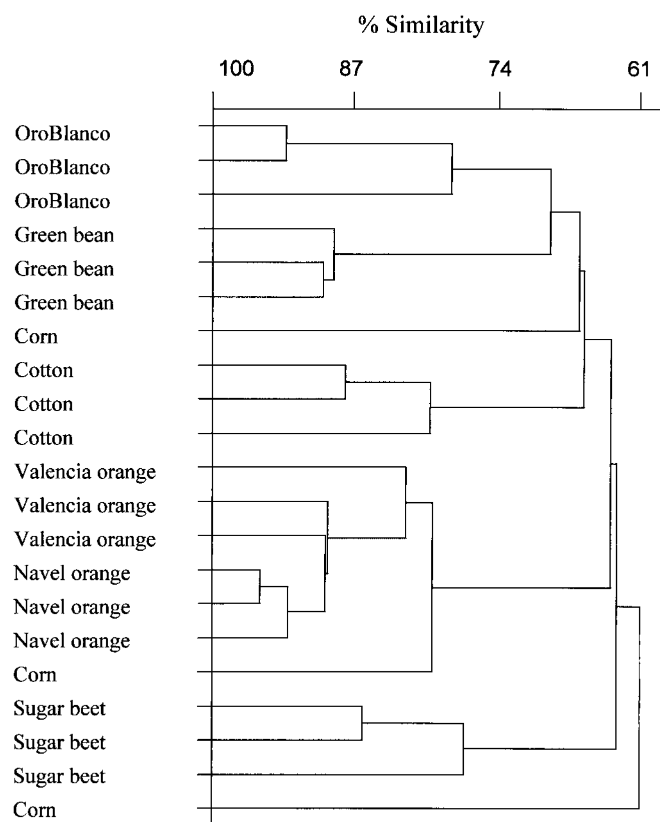

Fig. 2. Cluster analysis of 16S rDNA banding profiles for epiphytic bacteria from the phyllosphere of OroBlanco, Valencia orange, navel orange, cotton, corn, sugar beet, and green bean.

mined by using the single linkage method with Euclidean distance measure for determination of differences between clusters.

rRNA Gene Analysis. DNA bands from DGGE were sliced out and placed into a vial containing 20 μl of sterilize distilled water. The vials were kept at 4°C overnight to allow passive diffusion of DNA into the water and 10 μl of the eluted rDNAs were further amplified by using the bacterial universal primers described above. The resulting PCR products were purified from a 2% agarose gel and cloned into the pGEM-T Easy vector (Promega). The 200-bp rDNA inserts in pGEM-T were sequenced with an Applied Biosystems Prism 377 DNA sequencer using universal M13/pUC forward and reverse primers (GIBCO/BRL, Rockville). Microbe rDNA sequences were analyzed with blast searches (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). The overall similarities of small subunit rDNA sequences to described bacteria in the databases were determined by using the program PRETTY (SeqWeb Version 1.1, Genetics Computer Group, Madison, WI).

Results

Microbial Community Structure on the Phyllosphere of Different Plant Species. By using DGGE analysis of microbes released from whole leaves by sonication, distinct 16S rDNA banding patterns were observed from different plants (Fig. 1). The banding profiles were analyzed by using cluster analysis to examine the relative similarities of bacterial communities on the phyllosphere of each crop. Within the citrus group, similar community structures were observed in navel and Valencia oranges (Fig. 2). The phyllosphere community profiles from three OroBlanco trees clustered as their own group but separated from the other orange cultivars. The DNA banding profiles from replicate OroBlanco, green bean, cotton, Valencia orange, navel orange, and sugar beet samples were clustered, indicating distinct selection pressures on the epiphytic microorganisms of these plant species. An exception was corn, for which the communities were dissimilar in the three phyllosphere samples collected (Fig. 2).

Comparison of Phyllosphere Bacterial Communities as Determined by Culture-Independent Analysis and BIOLOG Plates. Distinct banding profiles were detected between epiphytic microorganisms directly extracted from the leaf surface as compared with those that grew in BIOLOG wells (Fig. 3). When BIOLOG substrate utilization patterns were examined, pyruvic acid methyl ester, D-mannitol, N-acetyl-D-glucosamine, glucose-1-phosphate and α-D-lactose showed redox dye color changes with the phyllosphere samples of all three Valencia trees. When DNA from these BIOLOG wells was extracted and analyzed on DGGE gels, distinct 16S rDNA banding profiles were observed from BIOLOG wells with the same carbon source but from different tree samples. This indicated that epiphytic microbial communities may be plant specific. In contrast, similar DGGE banding profiles (lanes E2, G2, and H1 and lanes E10 and G10) were observed from wells containing different carbon sources from the same phyllosphere samples.

Identification of Dominant Epiphytic Bacterial Communities. To further understand differences between the culture-independent and BIOLOG analysis of epiphytic bacteria, dominant 16S rDNA DGGE bands directly obtained from Valencia orange leaves (culture independent) or from BIOLOG wells (culturable) were isolated, cloned, and sequenced (Fig. 3). Four major dominant DGGE bands from the culture-independent analysis were selected (Fig. 3*A*, lane Val3). The nucleotide sequences of five individual clones were obtained from each of these bands (Table 1). Compared with gene databases, five out of the seventeen bacterial 16S rDNA fragments sequenced had DNA sequence similarity less than 90% to described microbial species. Only four species discovered here, *Acinetobactor* sp., *Bacillus pumilus*, *Enterobacter agglomerans*, and unidentified Cytophagales, had been previously reported in the phyllosphere (8–10, 12, 28). The *Bacillus pumilus* and unidentified Cytophagales sequences from the Valencia orange leaf samples had only 94% and 92% small subunit sequence similarity to previously described bacteria (GenBank accession nos. AB020208 and AF078327). Sequence data from clones isolated from the four major 16S rDNA DGGE bands therefore indicated that more than one microorganism was presented by each single band. Three eukaryotic sequences (homologous to GenBank sequences M55639, U43465, and U42624), located at band position 1, were also identified in the DGGE gels using primers PRBA338f and PRUN518r (27).

The epiphytic microorganisms that grew in BIOLOG wells gave dominant 16S rDNA DGGE banding patterns that were much different from those obtained by the culture-independent method. Dominant DGGE bands in the BIOLOG wells from eight major carbon sources (pyruvic acid methyl ester, D-mannitol, N-acetyl-D-glucosamine, glucose-1-phosphate, α-D-lactose, L-asparagine, D-galacturonic acid, and D-galactonic acid γ-lactone) were recovered for sequence analysis (Fig. 3 *A* and *C*). Nucleotide sequences were determined for five individual clones from each selected DGGE band, including 16S rDNA bands that had similar electrophoretic distances to the DGGE bands observed from the culture independent analysis of the Valencia orange phyllosphere samples. Unlike the culture-independent analysis, in which several rDNA sequences were represented in a single dominant band, only one rDNA sequence per dominant band

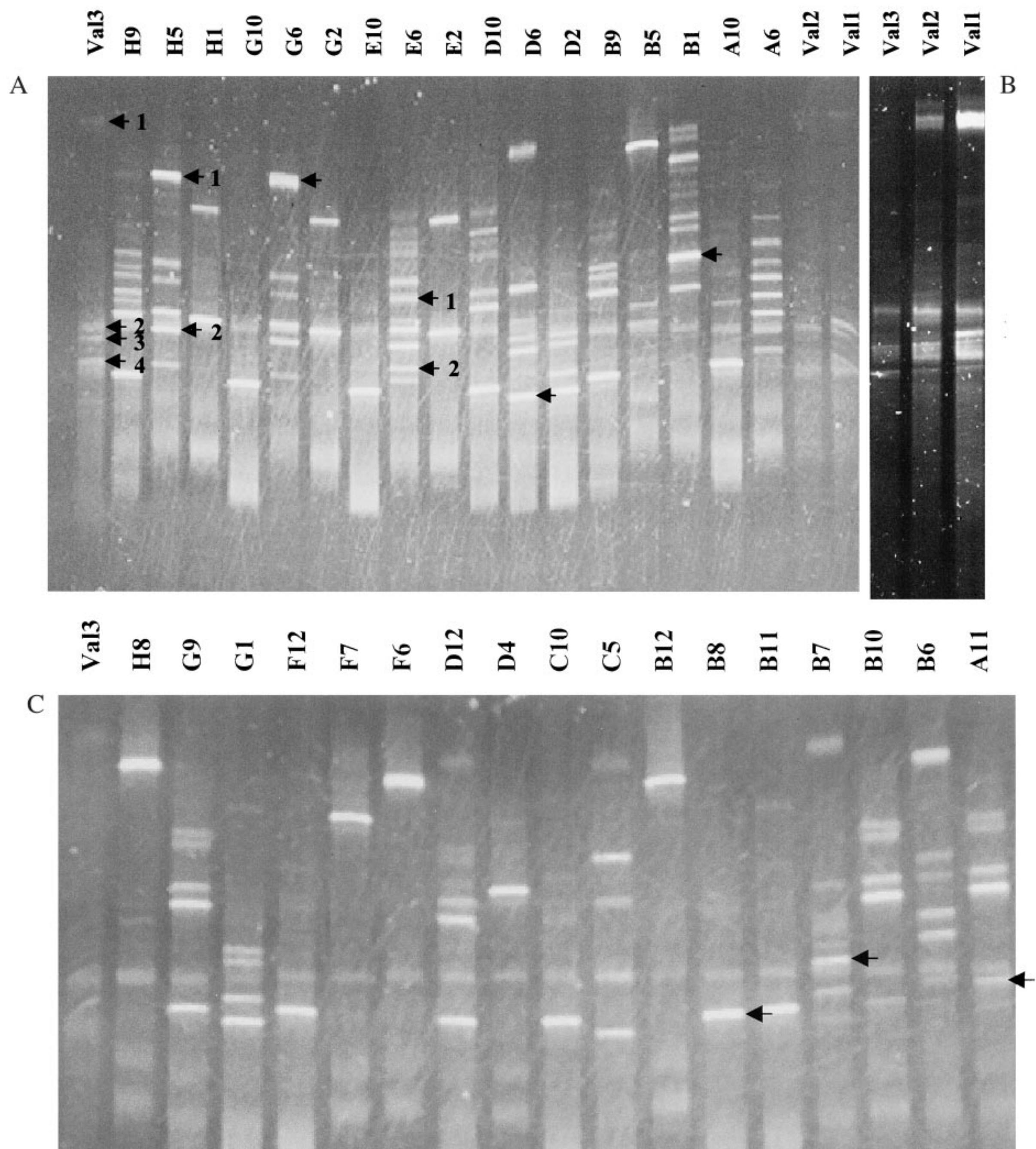

Fig. 3. (*A*) PCR DGGE 16S rDNA banding profiles of epiphytic bacteria on citrus Valencia trees either directly extracted for DGGE or extracted after 4.5 days growth in different wells of a BIOLOG plate at 25°C. Lanes Val1, Val2, and Val3 are epiphytic bacteria directly extracted from Valencia orange trees 1, 2, and 3. Lanes: A6 and A10, BIOLOG with β-methyl-D-glucoside; B1, B5, and B9, BIOLOG with pyruvic acid methyl ester; D2, D6, and D10, BIOLOG with D-mannitol; E2, E6, and E10, BIOLOG with N-acetyl-D-glucosamine; G2, G6, and G10, BIOLOG with glucose-1-phosphate; H1, H5, and H9, BIOLOG with α-D-lactose. Lanes B1, D2, E2, G2, and H1 were samples from Valencia tree 1. Lanes A6, B5, D6, E6, G6, and H5 were samples from Valencia tree 2. Lanes A10, B9, D10, E10, G10, and H9 were from Valencia tree 3. (*B*) PCR DGGE 16S rDNA banding profiles of epiphytic bacteria from citrus Valencia leaves directly extracted for DGGE. Lanes Val1, Val2, and Val3 are epiphytic bacteria directly extracted from Valencia orange trees 1, 2, and 3. (*C*) PCR DGGE 16S rDNA banding profiles of phylloplane bacteria directly extracted from Valencia trees or extracted after 4.5 days growth in different wells of a BIOLOG plate at room temperature. Lanes: A11, BIOLOG with D-galactonic acid γ-lactone; B6 and B10, BIOLOG with D-xylose; B7 and B11, BIOLOG with D-galacturonic acid; B8 and B12, BIOLOG with L-asparagine; C5, BIOLOG with Tween 40; C10, BIOLOG with I-erythritol; D4 and D12, BIOLOG with L-serine; F6, BIOLOG with D-glucosaminic acid; F7, BIOLOG with itaconic acid; F12, BIOLOG with glycyl-L-glutamic acid; G1 and G9, BIOLOG with D-cellobiose; H8, BIOLOG with putrescine. Lanes D4 and G1 were samples from Valencia tree 1. Lanes B6, B7, B8, C5, F6, F7, and H8 were samples from Valencia tree 2. Lanes A11, B10, B11, B12, C10, D12, F12, and G9 were from Valencia tree 3. Lane Val3 is epiphytic bacteria directly extracted from Valencia orange trees 3.

was identified from the BIOLOG approach (Table 2). Also in contrast to the epiphytic bacteria directly extracted from the leaf surface and identified by the culture-independent method, the dominant organisms identified from BIOLOG plates all showed a high similarity of 16S rDNA sequences (97–100%) to bacteria frequently described in the literature.

Table 1. Microorganisms identified from predominant 16S rDNA DGGE bands obtained from the phyllosphere of citrus Valencia leaves

Database match (GenBank accession no.)	Sequence similarity, %	Gel position	GenBank accession no.
Aureobasidium pullulans 16S-like ribosomal gene (M55639)	100	Band 1	AF231471
Lewia infectoria small subunit rRNA gene (U43465)	100	Band 1	AF231472
Morchella esculenta 18S small subunit rRNA gene (U42624)	100	Band 1	AF231473
Acinetobacter sp. (Z93446)	100	Band 1	AF231474
Desulfurominas choroethenica (U49748)	86	Band 1	AF231475
Spingomonas adhaesiva (D13722)	99	Band 2	AF231476
Uncultured delta proteobacerium (AJ241001)	87	Band 2	AF231477
Uncultured bacterium (AJ009487)	89	Band 2	AF231478
Bacillus pumilus (AB020208)	94	Band 2	AF231479
Clostridium bifermentans (X73437)	100	Band 2	AF231480
Uncultured delta proteobacterium (AJ241001)	100	Band 3	AF231481
Marinobacter hydrocarbonoclasticus (AB019148)	95	Band 3	AF231482
Unidentified Cytophagales (AF078327)	92	Band 3	AF231483
Uncultured delta proteobacterium (AJ241001)	84	Band 3	AF231484
Uncultured bacterium (AJ009475)	88	Band 4	AF231485
Enterobacter agglomerans (AF157688)	100	Band 4	AF231486
Unclassified organism 16S rRNA gene (X97111)	90	Band 4	AF231487

Discussion

Although our focus was narrow and the results not exhaustive, a culture-independent method disclosed a very different representation of the phyllosphere microflora from seven plant species than did a culture-based method. Strikingly, most of the phyllosphere microorganisms identified from dominant 16S rDNA sequences using DGGE on directly extracted leaf surface microorganisms had not been described in previous culture-based studies (8–10). While surprising, given the considerable literature on phyllosphere biology (1, 3–6, 8–10, 12, 28), the results are consistent with observations for other ecosystems in which culture-independent methods have shown much greater complexity than culture-dependent methods. For example, a PCR-amplified 16S rDNA clone library of marine bacteria attaching to the leaf surface of *Halophila stipulacea* was constructed (29). From a total of 60 rDNA clones sequenced, most of the sequences displayed less than 95% homology to known rDNA sequences. In a study of wall paint degraders, different dominant bacteria were also observed by using agar plating and DGGE methods (30), suggesting that the cultured organisms from agar plates were in fact not the predominant species. Some numerically dominant bands from the potato rhizosphere community were also not represented in the temperature gradient gel electrophoresis (TGGE) profiles of any of the BIOLOG wells (14). Bacterial communities from historical window glass were also analyzed by using DGGE of PCR-amplified 16S rDNA fragments (31) and were demonstrated to be much more complex than previously believed.

There are several reasons why distinct and simpler community structures were observed with phyllosphere samples analyzed by culture in BIOLOG wells as compared with the culture-independent method. These include different nutritional requirements, generation times, and antagonistic/synergistic interactions among phyllobacteria. Although the carbon metabolizing profiles in BIOLOG wells are often used as a reflection of the catabolic potential of a community (14, 32, 33), these culture conditions clearly do not reflect the epiphytic microbial community *in situ*. Indeed, our DGGE results indicated that different subsets of phyllosphere communities became dominant in the BIOLOG wells through enrichment, such that fewer bacteria were present in each dominant DGGE band sequenced. In 10 dominant DGGE bands from eight major carbon sources on which bacteria grew, only *Acinetobacter hemolyticus* (which grew in α-D-lactose) and *Enterobacter asburiae* (which grew in D-galacturonic acid) were also identified by direct DGGE analyses of rDNA from leaf microorganisms. The dominant bacteria found in BIOLOG wells in our study (*Acinetobacter hemolyticus*, *Pseudomonas oleovorans*, *P. putida*,

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Table 2. Bacterial isolates identified via predominant 16S rDNA DGGE bands from phyllosphere samples of citrus Valencia leaves incubated for 4.5 days in BIOLOG EcoPlates containing different carbon sources

Database match (GenBank accession no.)	Sequence similarity, %	Carbon source	GenBank accession no.
Pseudomonas oleovorans (D84018)	100	Pyruvic acid methyl ester (B1)	AF231488
Erwinia herbicola (AB004757)	100	D-Mannitol (D6)	AF231489
Erwinia amylovora (AJ233410)	98	*N*-Acetyl-D-glucosamine (E6) band 1	AF231490
Erwinia amylovora (AJ233410)	97	*N*-Acetyl-D-glucosamine (E6) band 2	AF231491
Pseudomonas putida (D85993)	100	Glucose-1-phosphate (G6)	AF231492
Acinetobacter haemolyticus (Z93436)	100	α-D-Lactose (H5) band 1	AF231493
Acinetobacter haemolyticus (Z93436)	99	α-D-Lactose (H5) band 2	AF231494
Erwinia herbicola (AB004757)	97	L-Asparagine (B8)	AF231495
Erwinia rhapontici (U80206)	98	D-Galactonic acid γ-lactone (A11)	AF231496
Enterobacter asburiae (AB004744)	98	D-Galacturonic acid (B7)	AF231497

The two sequences obtained for *Erwinia amylovora* and *Acinetobacter haemolyticus* are assumed to represent different rDNA operons from the same bacteria.

Erwinia amylovora, *E. herbicola*, *E. rhapontici*, and *Enterobacter asburiae*) have been commonly described as epiphytes from the phyllosphere by using cultural methods (8–10). As observed in studies with other ecosystems, BIOLOG analysis skewed the observed microorganisms to the proteobacteria, relative to the culture-independent method.

The sequence similarity of 16S rDNA fragments from bacteria enriched in BIOLOG plates were highly similar (97–100%) to described database bacteria. In marked contrast, of the 17 dominant phyllobacteria directly detected by DGGE, only four of them had 16S rDNA sequences between 96% and 100% similar to previously described bacteria (9, 10, 12, 28). Another five species had sequence similarities lower than 90%. These latter phyllosphere bacteria may not have been previously reported in nature.

Although leaf surface topography and nutrients present on the leaf surface are generally recognized as important regulators of phyllosphere microbial communities, little research has been done at the whole community level (8). By using *Solanum tubersum* L., highly similar PCR-DGGE fingerprints of bacterial phyllosphere communities were found on wild-type and T4-lysozyme transgenic variants (12). Out of seven different plants tested in this study, six of them showed a common and distinctive bacterial community. Among the citrus group, a higher similarity of the bacterial communities was observed between Valencia and navel orange, as might be expected because these cultivars were derived from a single ancestor by mutation (34). Oro-Blanco, which showed a distinct microbial community structure, is a hybrid of pumelo [*C. maxima* (Burm.) Merrill] and grapefruit (*C. paradis* Macf.), and it is quite divergent from Valencia and navel oranges. This is particularly noteworthy because the grapefruit and Valencia orange trees were in the same plot. Diverse phyllosphere community profiles were only observed with different phyllosphere samples from corn. The reason for this remains to be addressed, but it may be related to the rapid growth of these annual plants. Although more work is required to establish significance, it is nonetheless interesting that the culture-independent DGGE band profiles from Valencia citrus varied considerably between leaf samples harvested about 1 year apart (Figs. 1 and 3).

Despite its greater resolving power as compared with culture methods, several limits and cautions were noted concerning the use of DGGE for community analysis of leaf phyllosphere microorganisms. As reported by other researchers, rDNA bands generally reflect the presence of multiple microbial species, indicating limits of resolution when applying DGGE to complex environmental samples (12, 14, 25, 35). Because of the heterogeneity of small subunit rDNA operons in a single bacterial strain, an overestimate of populations may also occur if more than one DGGE band is amplified from a single strain. Although the direct DGGE method appears to reflect the *in situ* phyllosphere community with greater fidelity than culture-based methods, microorganisms that are not efficiently released from the leaf surface by sonication might be missed by the technology used. Finally, DGGE analysis tends to reveal only the dominant microflora, a serious deficiency assuming that minor organisms may make important biological contributions to an ecosystem. We are currently addressing these issues by investigating alternative methods for characterization of phyllosphere communities such as microarray technology and automatic ribosomal intergenic spacer analysis (36).

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1. Beattie, G. A. & Lindow, S. E. (1999) *Phytopathology* **89,** 353–359.
2. Pattantyus, J. & Kiss, S. (1994) *Stud. Univ. Babes-Bolyai Biol.* **39,** 97–103.
3. Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F. & Kloepper, J. W. (1997) *Can. J. Microbiol.* **43,** 895–914.
4. Andrews, J. H., Kenerley, C. M. & Nordheim, E. V. (1980) *Microb. Ecol.* **6,** 71–84.
5. Kinkel, L. L., Wilson, M. & Lindow, S. E. (1996) *Appl. Environ. Microbiol.* **62,** 3413–3423.
6. O'Brien, R. D. & Lindow, S. E. (1989) *Phytopathology* **79,** 619–627.
7. Wilson, M. & Lindow, S. E. (1994) *Appl. Environ. Microbiol.* **60,** 4468–4477.
8. Hirano, S. S. & Upper, C. D. (2000) *Microbiol. Mol. Biol. Rev.* **64,** 624–653.
9. Legard, D. E., McQuilken, M. P., Whipps, J. M., Fenlon, J. S., Fermor, T. R., Thompson, I. P., Bailey, M. J. & Lynch, J. M. (1994) *Agric. Ecosyst. Environ.* **50,** 87–101.
10. Thompson, I. P., Bailey, M. J., Fenlon, J. S., Fermor, T. R., Lilley, A. K., Lynch, J. M., McCormack, P. J., McQuilken, M. P. & Purdy, K. J. (1993) *Plant Soil* **150,** 177–191.
11. Cirvilleri, G. & Caldarera, G. (1998) *Z. Pflanzenkr. Pflanzenschutz* **105,** 441–451.
12. Heuer, H. & Smalla, K. (1999) *FEMS Microbiol. Ecol.* **28,** 357–371.
13. Goodfriend, W. L. (1998) *Soil Biol. Biochem.* **30,** 1169–1176.
14. Smalla, K., Wachtendorf, U., Heuer, H., Liu, W.-T. & Forney, L. (1998) *Appl. Environ. Microbiol.* **64,** 1220–1225.
15. Cattelan, A. J., Hartel, P. G. & Fuhrmann, J. J. (1998) *Soil Sci. Soc. Am. J.* **62,** 1549–1555.
16. Denton, C. S., Bardgett, R. D., Cook, R. & Hobbs, P. J. (1999) *Soil Biol. Biochem.* **31,** 155–165.
17. Siciliano, S. D., Theoret, C. M., De Freitas, J. R., Hucl, P. J. & Germida, J. J. (1998) *Can. J. Microbiol.* **44,** 844–851.
18. Torsvik, V., Daae, F. L., Sandaa, R.-A. & Ovreas, L. (1998) *J. Biotechnol.* **64,** 53–62.
19. van Hannen, E. J., Zwart, G., Van Agterveld, M. P., Gons, H. J., Ebert, J. & Laanbroek, H. J. (1999) *Appl. Environ. Microbiol.* **65,** 795–801.
20. Balkwill, D. L., Murphy, E. M., Fair, D. M., Ringelberg, D. B. & White, D. C. (1998) *Microb. Ecol.* **35,** 156–171.
21. Mahaffee, W. F. & Kloepper, J. W. (1997) *Microb. Ecol.* **34,** 210–223.
22. Pennanen, T., Fritze, H., Vanhala, P., Kiikkila, O., Neuvonen, S. & Baath, E. (1998) *Appl. Environ. Microbiol.* **64,** 2173–2180.
23. Sicliano, S. D. & Germida, J. J. (1998) *Soil Biol. Biochem.* **30,** 1717–1723.
24. Steinberger, Y., Zelles, L., Bai, Q. Y., Von Luetzow, M. & Munich, J. C. (1999) *Biol. Fertil. Soils* **28,** 292–300.
25. Yang, C.-H. & Crowley, D. E. (2000) *Appl. Environ. Microbiol.* **66,** 345–351.
26. Zwart, G., Huismans, R., Van Agterveld, M. P., Van De Peer, Y., De Rijk, P., Eenhoorn, H., Muyzer, G., Van Hannen, E. J., Gons, H. J. & Laanbroek, H. J. (1998) *FEMS Microbiol. Ecol.* **25,** 159–169.
27. Øvreås, L., Forney, L., Daae, F. L. & Torsvik, V. (1997) *Appl. Environ. Microbiol.* **63,** 3367–3373.
28. Dickinson, C. H., Austin, B. & Goodfellow, M. (1975) *J. Gen. Microbiol.* **91,** 157–166.
29. Weidner, S., Arnold, W., Stackebrandt, E. & Pühler, A. (2000) *Microb. Ecol.* **39,** 22–31.
30. Roelleke, S., Witte, A., Wanner, G. & Lubitz, W. (1998) *Int. Biodeterior. Biodegrad.* **41,** 85–92.
31. Roelleke, S., Gurtner, C., Drewello, U., Drewello, R., Lubitz, W. & Weissmann, R. (1999) *J. Microbiol. Methods* **36,** 107–114.
32. El Fantroussi, S., Verschuere, L., Verstraete, W. & Top, E. M. (1999) *Appl. Environ. Microbiol.* **65,** 982–988.
33. Garland, J. L. (1997) *FEMS Microbiol. Ecol.* **24,** 289–300.
34. Fang, D. Q. & Roose, M. L. (1997) *Theor. Appl. Genet.* **95,** 408–417.
35. Muyzer, G., de Waal, E. C. & Uitterlinden, A. G. (1993) *Appl. Environ. Microbiol.* **59,** 695–700.
36. Fisher, M. M. & Triplett, E. W. (1999) *Appl. Environ. Microbiol.* **65,** 4630–4636.