Culture-dependent and culture-independent assessment of bacteria in the apple phyllosphere

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Abstract

Aims: Bacterial communities in the apple phyllosphere were examined quantitatively and qualitatively by applying culture-dependent and culture-independent methods.

Methods and Results: Populations estimated by viewing cells stained with 4',6-diamidino-2-phenylindole generally were at least 100–1000 times greater than populations estimated by culturing on tryptic soy agar (TSA). Of the 44 operational taxonomic units (OTUs; cut-off threshold of 97%) detected in total, five bacterial orders containing 23 OTUs were identified by culturing on TSA, whereas nine orders containing 33 OTUs were identified by 16S rRNA gene cloning of DNA extracted from apple leaf surfaces. Twelve of the 44 OTUs were shared between cultured isolates and 16S rRNA gene clones and included the orders Burkholderiales, Pseudomonadales, Rhizobiales and Sphingomonadales. Three OTUs within the genus Sphingomonas accounted for 40% of isolates and 68% of clones. The Actinomycetales were found only among isolates, whereas the Bacteroidales, Enterobacterales, Myxococcales and Sphingobacteriales were represented in the 16S rRNA gene clone libraries but were absent among isolates.

Conclusions: Culture-independent methods revealed greater numbers and greater richness of bacteria on apple leaves than found by culturing.

Significance and Impact of the Study: This is the first study to directly compare culture-dependent and independent approaches for assessing bacterial communities in the phyllosphere. The biases introduced by different methods will have a significant impact on studies related to phyllosphere ecology, biological control of plant diseases, reservoirs of antibiotic resistance genes and food safety.

Introduction

Bacterial communities in the apple phyllosphere have been studied to further knowledge of the ecology and biological control of Erwinia amylovora, a Gram-negative bacterium that causes fire blight disease (Johnson and Stockwell 2000; Lindow and Brandl 2003). Additional studies of bacteria in the apple phyllosphere have focused on bacterial resistance to streptomycin and oxytetracycline, antibiotics used to protect apple, pear and certain other rosaceous plants from infection by Erw. amylovora (Norelli et al. 1991; Sobiczewski et al. 1991; Burr et al. 1993; Chiou and Jones 1993; Huang and Burr 1999; Schnabel and Jones 1999). In all cases, these studies have relied on first culturing bacteria from apple leaves prior to identification based on morphological and physiological tests. However, culturing potentially eliminates slower-growing bacteria and those unable to grow on the test media.

Culture-independent methods have been used extensively to study bacteria in soil, water and the plant rhizosphere (Basiliko et al. 2003; Fierer and Jackson 2006; Janssen 2006; Pham et al. 2008; Buée et al. 2009). However, their use to study phyllosphere microbial ecology is...
relatively recent, and primary literature in this area of research is still sparse. Using culture-independent denaturing gradient-gel electrophoresis (DGGE) on the 16S rRNA gene, Yang et al. (2001) showed that microbial community structure was similar on leaves of different individuals within a plant species but different across plant species. Furthermore, DGGE analysis revealed sequences of bacteria previously not known to inhabit plant leaves and five sequences presumed to represent sequences of bacteria previously not known to inhabit plant species. Furthermore, DGGE analysis revealed sequences of bacteria previously not known to inhabit plant leaves and five sequences presumed to represent bacteria not previously described. By contrast, bacteria cultured from leaves had rRNA gene sequences that were 97–100% similar to rRNA genes of previously described phyllosphere bacteria. Lambais et al. (2006) analysed 16S rRNA gene clone libraries constructed from DNA washed from leaves of tropical trees and reported that only 0–5% of the bacterial species were common to all trees under study, and 97% of the sequences represented species not previously described. Jackson et al. (2006) showed that there was little overlap in the bacterial community memberships in the phyllosphere of resurrection fern in two different physiological states, and none of the cloned 16S rRNA gene sequences were closely related to 16S rRNA genes of known phyllosphere bacteria. In the only previous report of culture-independent assessment of apple phyllosphere bacteria, Ottesen et al. (2009) analysed 16S rRNA clone libraries and found similar bacterial communities on leaves of apple trees under organic or conventional management regimes.

Despite these recent advances, there are few reports in which bacterial communities have been characterized by applying culture-dependent and culture-independent methods in parallel to the same samples. Moreover, we are aware of no such studies conducted on bacterial communities on leaf surfaces. Therefore, the aim of the current study was to compare bacterial communities in the apple phyllosphere using culture-dependent and culture-independent methods. Specifically, we compared bacterial populations determined by counting colony-forming units (CFU) on a solid medium with populations determined by counting bacterial cells washed from apple leaves and stained with 4′,6-diamidino-2-phenylindole (DAPI). We then compared the diversity of the bacterial community cultured from apple leaves with the community represented in culture-independent 16S rRNA gene clone libraries constructed from DNA isolated from apple leaves.

Materials and methods

Site descriptions

Apple leaf samples were collected in southern Wisconsin at five commercial orchards (Ep, OW, EL, BW and DC) and a research orchard (WM) at the University of Wisconsin West Madison Agricultural Research Station. The relative distances of Ep, OW, EL, BW and DC to WM, the research orchard, are 10, 64, 112, 121 and 26 km, respectively. At all sites, fungicides, insecticides and fertilizers were applied as needed to maintain tree health. In addition, trees at the commercial orchards Ep and OW were sprayed with streptomycin sulfate at a concentration of 50–100 μg ml⁻¹ 1–3 times during bloom (early to mid May) each year since at least 1997 to control fire blight.

Sampling

At orchards Ep and WM, 150 leaves from cultivar Gala were picked arbitrarily from a group of six trees on July 25 (Ep) and September 7 (WM), 2006. Samples were collected at least 2 days after the last rainfall to minimize the impact of rain on bacterial community structure (Hirano and Upper 1990). The leaves were handled with gloved hands by their petioles, bulked in batches of 50 leaves in sterile bags, transported to the laboratory on ice and stored at 4°C until processed. All samples were processed within 2 days of collection. Using sterile tweezers and scissors, petioles were removed and discarded. Each leaf blade was weighed individually, and then groups of three leaves were sonicated in a tabletop ultrasonic cleaning bath (Branson model 3510 (Branson Ultrasonics Corp., Danbury, CN)) for 5 min in 50 ml of sterile deionized water containing 2–3 drops of Tween 20 per litre. The extracts of all the leaves from a single site were bulked and then passed through a 10-μm nylon filter to exclude debris.

In separate experiments in 2004 through 2006, 5–10 leaves were collected from each of six orchards during June through September. Leaves were collected from cultivars Cortland, Empire, Gala, and Golden Delicious (Table 1). Each leaf was sonicated individually in water and subsequent steps were as described earlier.

Bacterial enumeration

An aliquot of the filtered leaf extract from each orchard site (bulked samples) or from each individual leaf samples was serially diluted and then plated onto 0.1x tryptic soy agar (TSA) amended with cycloheximide (100 μg ml⁻¹) to inhibit fungal growth. After 7 days of incubation in the laboratory at ambient temperature and light, the bacterial colonies were viewed under a stereomicroscope and counted.

A separate aliquot of the filtered leaf extract was fixed in formaldehyde and stained with DAPI. For each sample, stained cells were viewed by microscopy (Olympus BW60 equipped with a 100 W Hg arc lamp, with 365 ± 10-nm excitation and 450–475-nm bandpass emission filters; Olympus America Inc., Center Valley, PA) and counted.
Table 1 Comparison of bacterial populations from apple leaves estimated by colony-forming units and direct cell count

<table>
<thead>
<tr>
<th>Sampling date</th>
<th>Site name</th>
<th>Cultivar</th>
<th>Log$_{10}$ CFU per gram of leaf (SE)*</th>
<th>Log$_{10}$ direct cell count per gram of leaf (SE)†</th>
<th>P-value from paired t-test†</th>
</tr>
</thead>
<tbody>
<tr>
<td>July 27, 2004</td>
<td>OW</td>
<td>Cortland</td>
<td>6.39 (0.12)</td>
<td>9.12 (0.06)</td>
<td>&lt;0.001</td>
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<tr>
<td>September 21, 2004</td>
<td>EL</td>
<td>Golden Delicious</td>
<td>7.23 (0.06)</td>
<td>9.55 (0.05)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>September 21, 2004</td>
<td>BW</td>
<td>Golden Delicious</td>
<td>7.28 (0.86)</td>
<td>9.23 (0.19)</td>
<td>0.001</td>
</tr>
<tr>
<td>September 21, 2004</td>
<td>DC</td>
<td>Empire</td>
<td>6.89 (0.28)</td>
<td>9.38 (0.10)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>June 20, 2006</td>
<td>WM</td>
<td>Gala</td>
<td>3.87 (0.16)</td>
<td>8.48 (0.07)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>June 30, 2006</td>
<td>WM</td>
<td>Gala</td>
<td>4.70 (0.38)</td>
<td>9.00 (0.09)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>July 6, 2006</td>
<td>WM</td>
<td>Gala</td>
<td>4.44 (0.11)</td>
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<td>WM</td>
<td>Gala</td>
<td>4.04 (0.11)</td>
<td>9.12 (0.16)</td>
<td>&lt;0.001</td>
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<td>July 12, 2006</td>
<td>Ep</td>
<td>Gala</td>
<td>6.14 (0.32)</td>
<td>9.20 (0.05)</td>
<td>0.001</td>
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<td>July 17, 2006</td>
<td>WM</td>
<td>Gala</td>
<td>5.30 (0.48)</td>
<td>9.11 (0.07)</td>
<td>0.001</td>
</tr>
<tr>
<td>July 17, 2006</td>
<td>Ep</td>
<td>Gala</td>
<td>6.71 (0.15)</td>
<td>9.39 (0.04)</td>
<td>&lt;0.001</td>
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<tr>
<td>July 25, 2006</td>
<td>Ep</td>
<td>Gala</td>
<td>7.46</td>
<td>10.29</td>
<td>NA§</td>
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<tr>
<td>September 7, 2006</td>
<td>WM</td>
<td>Gala</td>
<td>7.88</td>
<td>8.11</td>
<td>NA§</td>
</tr>
</tbody>
</table>

SE, standard error of the mean is in parentheses

*CFU, colony-forming units on 0.1% tryptic soy agar after 7 days.
†Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) and then counted by microscopy.
§P-values not available because leaf samples were bulked rather than tested individually.

in 10–25 fields of view to obtain total cell counts per gram of leaf. For the samples in which 5–10 replicate leaves were processed individually, a paired t-test was used to compare culture-dependent CFU counts and culture-independent DAPI counts.

DNA extraction and 16S rRNA gene library construction

From each of the bulked samples from orchards Ep and WM, approximately 300 bacterial colonies cultured on TSA were selected arbitrarily quadrant by quadrant from Petri plates containing the optimal cell dilutions. Colonies were streaked onto fresh TSA medium and then single isolates were suspended in sterile water and spreadplated. The resulting bacterial lawns were scraped, and using a random number generator, they were arrayed into sterile 96-well assay plates containing a 10% glycerol solution in 0.1% tryptic soy broth (TSB). The first 190 isolates were lysed in 50 μl water at 95°C for 10 min, and then 0.5 μl of the lysed suspension was added to a 40 μl PCR reaction containing bovine serum albumin (Cadillo-Quiroz et al. 2006) plus universal bacterial 16S ribosomal gene primers 27f (5′-AGA TTG TGA TCM TGG CTC AG) and 1492r (5′-TAC GGY TAC CTT GTT ACG ACT T) using the following program: 95°C for 4 min; 35 cycles of 95°C for 1 min, 50°C for 1 min, 72°C for 1-5 min; and a final elongation step at 72°C for 5 min. Primer 27f was then used to sequence from the 5′ end of the ribosomal gene.

For orchards Ep and WM, cells in the leaf extract remaining after bacterial enumeration were pelleted by centrifugation at 5500 g at 4°C for 20 min. The pellet plus approximately 4 ml of leaf extract was then transferred to microcentrifuge tubes and centrifuged at 16 870 g for 5 min to further concentrate bacteria. Genomic DNA was extracted using the FastDNAkit (MP Biomedicals, Solon, OH, USA), which includes a bead-beating step. For each genomic DNA extract, three PCRs were set up using primers 27f and 1492r as described previously (Bräuer et al. 2006). The amplification products were combined, purified and concentrated using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and then cloned into the pCR2.1 vector according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). Escherichia coli DH5α cells were transformed with the ligation products, and a clone library was constructed for each orchard site. One hundred ninety clones from each library were screened for the presence of appropriately sized inserts using the M13f(−20) and M13r(−27) primers according to previously described conditions (Bräuer et al. 2006). The directionality of the insert within the vector was determined using the M13f(−20) and 357f (CTC CTA CCG GAG GCA GCA G) primers with the following program: 95°C for 4 min; 35 cycles of 95°C for 1 min, 58°C for 1 min, 72°C for 15 min; and a final elongation step at 72°C for 5 min. Inserts were sequenced from the 5′ end using the appropriate M13 primer at the University of Wisconsin–Madison Biotechnology Center on an Applied Biosystems 3730xl automated DNA sequencing instrument (Applied Biosystems, Foster City, CA).
Bacterial communities

Phylogenetic analyses of bacterial communities

Sequences of the 16S rRNA gene from each isolate colony or clone were compared to the GenBank database using the local alignment tool, BLASTN (Altschul et al. 1990). The sequences were then aligned using the SILVA aligner (Pruesse et al. 2007) and imported into ARB (Ludwig et al. 2004) for further phylogenetic analyses. Potential chimeras were identified using Bellerophon (Huber et al. 2004) and Mallard (Ashelford et al. 2006) and omitted from further study after manual verification. For Bellerophon, the native aligner was used. For Mallard, the sequences were aligned with SILVA with the addition of 40 nearest neighbours, imported into ARB and exported after filtering out the nonaligning extremities of the sequences. Short sequences that did not cover the reference E. coli base positions 105–735 were also omitted from further study.

A neighbour-joining matrix with a Jukes–Cantor correction was generated in ARB from sequences from the Ep and WM isolate collections and Ep and WM clone libraries, and imported into DOTUR (Schloss and Handelsman 2005). DOTUR was used to assign operational taxonomic units (OTU) at the 97% sequence similarity level. The bacterial community structure was compared between cultured bacterial isolates and culture-independent clone libraries using SONS (Schloss and Handelsman 2006). The shared community membership was assessed using the Jaccard similarity indices. The OTU-based (sometimes called ‘classic’) Jaccard index determines the fraction of OTUs shared between communities (Schloss and Handelsman 2006), whereas the abundance-based Jaccard index determines the fraction of sequences that belong to OTUs found in both communities. The shared community structure, which takes into consideration both community membership and abundance, was examined using $\theta_{YC}$ values (Yue and Clayton 2005). Rarefaction curves and Shannon–Wiener and Simpson’s diversity indices were generated using mothur (Schloss et al. 2009).

To build a phylogenetic tree, a single representative sequence from each OTU from among all the isolates and clones was appointed. Well-characterized species were chosen as reference sequences from the SILVA database version 100 (Pruesse et al. 2007). A filter that excluded the nonoverlapping ends of the aligned sequences as well as insertions and deletions was applied before exporting the sequences from ARB. ModelTest was used to determine the best correction model, using the maximum likelihood–optimized base tree for likelihood calculations and Akaike Information Criterion for model selection (Posada 2008). PhyML was used to build a single phylogenetic tree of all the sequences (Guindon and Gascuel 2003). The settings used were as follows: the general time reversible correction model, empirical equilibrium frequencies, fixed transition/transversion ratio, estimated proportion of invariable sites, four substitution rate categories, an estimated gamma shape parameter, NNI type of tree improvement and 1000 bootstraps. The calculated tree was then split into two trees using TREEGRAPH2 for a more effective visual presentation (Stöver and Müller 2010). Tree topology was confirmed with trees constructed using maximum parsimony (ARB) and Bayesian algorithms (MrBayes3).

Nucleotide sequence accession numbers

The representative 16S rRNA gene sequences in this study were deposited in the GenBank database. The accession numbers for the bacterial isolates are HM989880–HM989893 and for the bacterial clones are HM450005–HM450035. The full library of sequences is available upon request.

Results

Bacterial enumeration

In all samples, more cells were detected per gram of leaf using the culture-independent approach of staining cells with DAPI than using the culture-dependent method of counting colonies (Table 1). Bacterial aggregates were not detected by microscopy. In cases where replicate sampling allowed statistical analysis, the differences were highly significant.

Diversity of cultured bacteria

The cultured community included members of the $\alpha$-, $\beta$-, and $\gamma$-Proteobacteria and the Actinobacteria (Table 2). Of the 309 isolates identified by 16S rRNA gene sequence, the predominant orders were Sphingomonadales and Actinomycetales, followed by the Rhizobiales, Pseudomonadales and Burkholderiales. With the exception of one isolate, all isolates were closely related to known bacteria. Ninety-seven per cent of the isolates were related at the species level (at least 97% sequence similarity) to bacteria previously described to inhabit the phyllosphere. The remaining 3% of the isolates were related to known phyllosphere bacteria by a similarity value of 95–96%. Plant hosts of these relatives varied and include soybean, clover, potato, bean, poplar, sunflower, Thlaspi, alpine subnival plant and grass. Some of the isolates were also close relatives of plant pathogens: Curtobacterium flaccumfaciens pv. flaccumfaciens, causal agent of bacterial wilt of bean; Pseudomonas syringae, causal agent of halo

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Table 2  Taxonomic distribution of operational taxonomic units (OTU) (species level) recovered from each isolate collection and clone library of each orchard site. OTUs were calculated at a threshold level of 97%. Genus assignment was based on a BLASTn identity of 95% or above with well-characterized species. The top BLASTn hit and per cent identity and coverage for each OTU were determined according to the respective representative sequence.

<table>
<thead>
<tr>
<th>Number of sequences</th>
<th>Orchard Ep</th>
<th>Orchard WM</th>
<th>Representative sequence</th>
<th>Accession number</th>
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<td></td>
<td>Isolates</td>
<td>Clones</td>
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bight of bean and several other plant diseases, as well as a common epiphyte and ice nucleator; and \textit{Xylophilus ampelinus}, causal agent of bacterial blight of grape. Ninety-eight per cent of the isolates were related at the species level to known air- and dust-borne bacteria. Ninety-six per cent of the isolates were related to both phyllosphere and air- or dust-borne bacteria at the species level and belonged to the genera \textit{Curtobacterium}, \textit{Frigobacterium}, \textit{Aeromicrobium}, \textit{Friedmaniella}, \textit{Frondihabitans}, \textit{Pseudomonas}, \textit{Methylobacterium}, \textit{Sphingomonas} and \textit{Novosphingobium}. The abundance at the species level of each OTU for each orchard site is listed in Table 2.

### Diversity within clone libraries

Cloned 16S rRNA gene sequences were from members of the \( \alpha \)-, \( \beta \)-, \( \delta \)- and \( \gamma \)-Proteobacteria and the Bacteroidetes (Table 2). Of the 317 clones examined, the predominant sequences were from bacteria in the order Sphingomonadales, followed by orders Pseudomonadales, Burkholderiales and Rhizobiales. The orders Bacteroidales, Myxococcales and Enterobacteriales were each represented by one clone. Aside from one clone, all clones in this study contained 16S rRNA genes with similarity of at least 96% to GenBank sequences. Four of the orders represented in clone libraries (Bacteroidales, Sphingobacteriales, Myxococcales and Enterobacteriales) were absent among cultured isolates, whereas the order Actinomycetales, which was prevalent among isolates, was not represented in clone libraries (Table 2; Fig. 1).

![Figure 1](image-url)
isolate sequences described earlier, plant hosts of these bacteria varied. Eleven per cent of the clones contained 16S rRNA genes from bacteria related either to airborne or phyllosphere organisms at the species level (97%). Among the remaining three per cent, clones representing Sphingomonas and Sphingobacteriales were related at the genus level to bacteria from dust, soil, phyllosphere and/or the human skin microbiome.

Rarefication analysis and richness estimates

The rarefaction curves for isolates and clones indicated that further sequencing would reveal additional unique OTUs (Fig. 2). The Shannon–Wiener index was $1.96 (95\% CI ±0.19)$ and $2.17 (95\% CI ±0.13)$, and the Simpson’s index was $0.32 (95\% CI ±0.06)$ and $0.17 (95\% CI ±0.02)$ for clones and isolates, respectively. The Shannon–Wiener indices indicated that the overall diversity among clones and isolates was similar, whereas the Simpson’s indices indicated that the clone libraries were significantly richer than isolate collections.

Bacterial community structure: cultured isolate vs cloned bacterial sequences

Among the 626 sequences from cultured isolates and clone libraries, 44 distinct OTUs were determined at the similarity threshold value of 97% (Table 2). Twelve of the 44 OTUs were shared between isolates and clones (Fig. 1). Sphingomonas was the dominant genus among the detected OTUs. Three OTUs within the genus Sphingomonas, represented by WM06x_A10C, Ep06x_A12B and Ep06x_A10D, encompassed 68% of clones and 40% of isolates (Table 2). These sequences were related by ≥97% to air- and dust-borne micro-organisms, such as Sphingomonas aurantiaca, and cloud-borne isolate PDD-14b-6, and by ≥98% to bacteria from various plants, mountain lakes and mire ponds, soils, human skin microbiome and alpine permafrost.

The OTU-based Jaccard similarity index of 0.27 between clones and isolates indicated that a relatively small number of OTUs was shared between them, as demonstrated in that only 12 of the 44 OTUs were shared (Fig. 1). The abundance-based Jaccard similarity index of $0.59 ± 0.18$ (SE) indicated that a relatively large number of sequences belonged to OTUs shared between clones and isolates. Indeed, 85% of cloned sequences and 65% of isolate sequences belonged to shared OTUs. The $\theta_YC$ value of $0.57 ± 0.06$ (SE) indicated that the community structures among clones and isolates differed. Notably, members of the phylum Bacteroidetes (includes orders Bacteroidales and Sphingomonadales) were represented in the clone libraries but not in the cultured isolates, totalling 52% of OTUs unique to clones (Fig. 1). Actinobacteria were present only among isolates, making up 64% of OTUs unique to isolates (Fig. 1). Furthermore, of the 22 cloned sequences representing the order Burkholderiales, only two shared a single common OTU with an isolate.

Phylogenetic trees constructed from a representative sequence of each OTU further illustrate differences in bacterial communities assessed by culture-dependent vs culture-independent methods (Fig. 3a,b). Within the Bacteroidetes, the clones representing Sphingobacteriales clustered together within a branch distinct from the reference strains. Indeed, clones belonging to both the Sphingobacteriales and Bacteroidales orders were most closely related (96–98%) to bacteria described as “uncultured” in GenBank (Table 2). Within the β-Proteobacteria, Acidovorax/Xylophilus were represented by isolates and clones, whereas Massilia spp. were represented only in clone libraries.

Figures 3 (a,b) Rooted maximum likelihood tree with 1000 bootstraps constructed for partial 16S rRNA gene from orchard bacterial community. A single representative sequence from each operational taxonomic unit (OTU) was selected for the phylogenetic tree (accession numbers HM450005-HM450035 and HM989880-HM989893). Bootstrap values above 50% are given at the branch nodes. Next to each representative sequence used in this study, the composition of the respective OTU is indicated by ● (clones) and ○ (cultured isolates). The letters Ep and WM represent the orchard of origin of the respective representative clone or isolate but does not indicate that the OTU is composed exclusively of sequences from any single orchard.
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(a)
Discussion

Many studies have demonstrated the power of culture-independent methods for revealing previously unappreciated microbial diversity (Yang et al. 2001; Lambais et al. 2006; Pham et al. 2008). However, our study is unique in that culture-dependent and culture-independent methods were compared directly (i.e. applied to the same samples) in a quantitative and qualitative assessment of bacterial communities on leaves. As such, the study highlights the influence that methods used to characterize bacterial communities have on results. With specific relevance to the apple phyllosphere, knowing the relative strengths and limitations of culture-dependent and culture-independent approaches will be important in studies focused on the impact of antibiotics used to control fire blight, biological control of fire blight and food safety. Furthermore, our study has revealed bacterial taxa not previously described in the apple phyllosphere (e.g. Myxococcales, Labdella and Terracoccus).

Cell counts determined by DAPI staining consistently showed at least 100–1000 times more bacterial cells on apple leaves than were estimated by culturing on 0·1·TSA, a medium commonly used to culture bacteria from environmental samples. These ratios, which we report for the first time for natural bacterial populations in the phyllosphere, corroborate what is typically observed in other environments, where it is estimated that the current culturing technology allows access to no more than 0·1–10% of the diversity (Handelsman and Smalla 2003). Despite that only a small fraction of the observed bacteria could be cultured on TSA, 85% of cloned sequences belonged to OTUs that were cultured, suggesting that as much as 85% of the total community should be culturable. A possible reason that a greater proportion of the bacterial community was not cultured may be because most cells were in the viable but nonculturable state or were dead. At the commercial orchards in our study, UV irradiation and desiccation, as well as exposure to bactericides such as copper and streptomycin, might have stressed bacteria in the phyllosphere. Environmental stresses and exposure to sublethal levels of antimicrobial chemicals can debilitate bacteria, making them unable to grow on media (Gauthier 2000; McFeters and LeChevallier 2000). Wilson and Lindow (1992) found that under conditions favourable for rapid growth, populations of Ps. syringae pv. syringae on bean leaves were similar whether determined by CFUs or microscopy. However, 80 h or later after inoculation, CFUs underestimated viable cell counts by two- to fourfold, leading the authors to specu-
late that discrepancies might be even greater in the field where bacteria are often not undergoing rapid multiplication. The use of other types of media or sampling during conditions that favour rapid bacterial multiplication might have narrowed the gap between the number of CFUs and number of bacteria counted by microscopy (Stevenson et al. 2004; Davis et al. 2005).

Another possible reason for the small fraction of culturable bacteria despite the large percentage of sequences shared between clones and isolates is that only a small percentage of the bacteria within the OTUs, determined at the 97% threshold level, are capable of growing under laboratory conditions. Indeed, microheterogeneity at either the rRNA gene or heterogeneity at the genomic level cannot be assessed by examining the rRNA gene at the 97% similarity threshold, and previous studies have shown that strains with identical 16S rRNA gene sequences can have radically different lifestyles (Taghavi et al. 1996; Jaspers and Overmann 2004).

Among apple leaf inhabitants, we identified five bacterial orders containing 23 OTUs by culturing on TSA compared to nine orders containing 33 OTUs by 16S rRNA gene cloning. Thus, the culture-independent assessment revealed greater richness than did culturing. The isolates, however, were not merely a subset of bacteria represented in clone libraries. For example, the order Actinomycetales, which was prominent in terms of both OTUs and sequences among isolates, was absent in clone libraries. This finding is consistent with the work of Ottesen et al. (2009) who found Actinobacteria represented by fewer than 3% of 16S rRNA gene sequences in libraries constructed from apple leaves. In general, the community composition of the clone libraries was similar to that previously reported in the apple phyllosphere (Ottesen et al. 2009), in that the most prevalent bacterial classes were the α-, β-, and γ-Proteobacteria, followed by the Sphingobacteria. It is difficult to know whether culturing selected for the Actinobacteria or whether steps in library construction selected against this group. Indeed, several studies have shown that DNA extraction and amplification procedures do affect the community structure described (Moré et al. 1994; Miller et al. 1999; Carrigg et al. 2007; Thakuria et al. 2008; McIlroy et al. 2009; Ning et al. 2009).

The larger number of OTUs obtained by cloning than by culturing was corroborated by a higher Simpson’s index for clones than isolates. However, the Shannon–Wiener index, which takes into account species evenness, was lower for clones than isolates. This is probably explained by the assignment of 55% of clone sequences to a single species of Sphingomonas. Compared to some previous studies conducted using culture-independent methods, the relatively low diversity among cloned sequences in our study may be because of filtering the apple leaf wash, a step intended to remove plant debris, which probably is the source of chloroplast contamination in 16S rRNA gene libraries constructed from phyllosphere DNA (E. Yashiro, unpublished data). In this study, very few clones contained chloroplast DNA, but the filtration step may have also excluded bacterial associates of eukaryotic residents (e.g. fungi, algae, insects) of the phyllosphere, thereby reducing diversity. The modest size of the clone libraries and culture collections, as well as bulking leaves during sampling, may have further contributed to the low overall diversity. The slopes of the rarefaction curves suggest that more OTUs would have been discovered with additional sampling of both clones and isolates. By bulking leaves, as opposed to sampling them individually, large populations of Sphingomonas spp. on a few leaves might have prevented the detection of more rare taxa.

The use of more than one type of growth media might have resulted in a more similar community structure between isolate collections and clone libraries. For instance, nutrient agar was found to enable growth of both type strains of Massilia aurea and Massilia brevitalea, which are closely related to clone sequences in our study (Gallego et al. 2006; Zul et al. 2008). TSA was reported to enable growth of M. aurea, although isolates of this genus were not identified in our study. Its absence in our culture collections may be because of the relatively small proportion of members of this genus within the phyllosphere community we sampled. Contrarily, the apple phyllosphere clone libraries contained many sequences within the phylum Bacteroidetes, a phylum absent from our isolate collections. In this case, the discrepancy in community structure between culture collections and clone libraries may have been narrowed had we used growth media such as R2A, a minimal medium often used to isolate and/or maintain novel species of Bacteroidetes (Kim et al. 2008; Klassen and Foght 2008; Zhang et al. 2009) in addition to TSA.

The majority of the bacterial sequences from both the isolate culture collections and clone libraries were similar to sequences from air- and dust-borne bacteria of various origins, including tropospheric clouds, airplane passenger cabin air, house dust and cowbarn air (Busse et al. 2003; Amato et al. 2007; Osman et al. 2008; Alenius et al. 2009). Interestingly, the majority of the bacteria in our study that were related to air- and dust-borne bacteria were also related to phyllosphere microbiota. Indeed, Lindemann et al. (1982) demonstrated that plants are a more important reservoir for airborne bacteria than is soil. Furthermore, Morris et al. (2008) showed that phyllosphere bacteria can travel great distances through the atmosphere before landing again on a plant host. In addition to corroborating previous observations, our
study provides evidence that the majority of the phylloplane bacterial taxa, and not just the culturable minority, are potentially well adapted to surviving the harsh conditions of an airborne existence. The predominance of Sphingomonas in our study is not surprising in the light of the findings that members of this genus scavenge a variety of substrates that are present in small quantities on leaves (Delmotte et al. 2009). Sphingomonas species have not been extensively studied for their role as epiphytes, despite their occurrence on leaves of diverse and economically important plants (Kim et al. 1998). Their inability to cause ice nucleation has been previously inferred by the fact that none of the 75 isolates from frost damaged vegetables were Sphingomonas species (Goto et al. 1989). Similarly, we found that none of 39 Sphingomonas isolates from apple leaves served as ice nucleators (data not shown).

Our study revealed no isolates and just one cloned sequence representing the genus Pantoea. This contrasts with previous studies in which Pantoea and the related enteric genera Erwinia and Enterobacter were commonly isolated from apple leaves (Schnabel and Jones 1999; Ottesen et al. 2009). Previous studies have shown a succession of culturable micro-organisms over the course of the growing season, with the more saprophytic species becoming more prevalent later in the season (Kinkel 1997). Thus, those members of the Enterobacteriaceae family might have been more predominant earlier in the growing season at sites Ep and WM, with populations subsiding by middle to late summer when samples were collected.

In summary, culture-independent microscopy and 16S rRNA gene libraries revealed greater numbers and greater richness of bacteria on apple leaves than found by culturing, despite a relatively modest number of sequences examined. Although there was significant overlap in the taxa identified by the two approaches, the strengths, limitations and biases of the different methods were highlighted.

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References


