Grapevine Microbiomics: Bacterial Diversity on Grape Leaves and Berries Revealed by High-Throughput Sequence Analysis of 16S rRNA Amplicons

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Abstract

We used the culture-independent method of high-throughput pyrosequencing of 16S ribosomal RNA (rRNA) gene amplicons to analyze the diversity of bacteria associated with the leaf and berry surfaces of 'Chardonnay' grape in a vineyard close to harvest. Combined, more than half of all bacterial sequences were classified as Proteobacteria. Other well-represented phyla were the Firmicutes, Bacteroidetes, and Actinobacteria. The most abundantly represented genera were *Sphingomonas*, *Hymenobacter*, *Bacillus*, *Pseudomonas*, *Skermanella*, *Leuconostoc*, *Massilia*, *Methylobacterium*, *Cellvibrio*, and *Curtobacterium*. Together, these top 10 genera accounted for almost one-third of all sequences. We observed differences in the composition of bacterial communities between leaves and berries. Members of the genera *Sphingomonas*, *Hymenobacter* and *Methylobacterium* were abundant on foliage, whereas *Achromobacter*, *Flavobacterium* and *Cellvibrio* were typical for the fruit. Moreover, certain *Pseudomonas* species appeared to associate exclusively with leaf surfaces, while others were found more frequently on berries. Unanticipated was the discovery of a novel Proteobacterium that dominated the bacterial community on berry surfaces. Our results form the basis for future investigations into the relationship between the structure of microbial communities on grape leaves and berries and the ecosystem (dis)services that these communities provide to grape growers at different pre- and post-harvest stages of production.

INTRODUCTION

The above-ground surfaces of plants are colonized in considerable numbers by many different microorganisms, including bacteria, fungi and yeasts (Leveau, 2009). Until recently, much of what was known about the microbial diversity associated with the phyllosphere (leaf surface) or carposhere (fruit surface) resulted from culture-dependent studies. Through techniques such as denaturing gradient gel electrophoresis and sequencing of 16S rRNA gene amplicons, it is now realized that these microbial communities (also known as microbiota) are more complex than should be assumed on the basis of culturable organisms alone (Heuer and Smalla, 1999; Yang et al., 2001; Handschur et al., 2005; Albino et al., 2006; Lambais et al., 2006; Delmotte et al., 2009; Redford et al., 2010; Hunter et al., 2011).

Presently, we are still far from a clear understanding of how factors such as the plant and the environment determine the composition of microbial communities on aerial plant surfaces. Even less is known about the impact of microbial community composition on the various ecosystem services and disservices that these communities provide. To illustrate this point, consider an economically important crop worldwide, grape (*Vitis vinifera*). Many of the pre- and post-harvest processes that are part of the production of wine, juice, table grapes or raisins are affected by or depend on microorganisms that are naturally associated with grape leaves and berries. For example, a pathogenic fungus such as *Erysiphe necator*, causative agent of grape powdery mildew, severely reduces yields in the vineyard (Pearson and Goheen, 2008), while spoilage bacteria such as *Acinetobacter*,

Lactobacillus, *Oenococcus*, and *Pediococcus* may interfere with the vinification process (Bartowski, 2008). Conversely, members of the grape-associated microbial community protect against establishment of foliar pathogens (Dimakopoulou et al., 2008), confer distinct aromas and flavors to the berries (Verginer et al., 2010), or reduce wine acidity (Bartowski, 2008). Many winemakers rely on the presence of naturally occurring or 'wild' yeasts on berry surfaces as catalysts of the sugar-to-ethanol conversion during fermentation (Varela et al., 2009).

Each of these important ecosystem (dis)services is performed by one or more members of the grape leaf or berry microbial community. The abundance and activity of these beneficial or deleterious members is a function of the interactions that these members have with others in the community. A better understanding of the microbial community composition may thus provide grape growers with more options for management of disease pressure in their vineyards and quality of their product, for example by early detection of a change in the community composition that is likely to favor the establishment of unwanted pathogenic fungi or desirable wild yeasts.

This study offers an early view into the bacterial diversity associated with grape leaves and berries. Our approach is based on the exploitation of culture-independent techniques, specifically the use of high-throughput pyrosequencing of 16S ribosomal RNA (rRNA) amplicons, to obtain a more inclusive picture of all types of bacteria that associate with the grape phyllo- and carposphere at the time of harvest.

MATERIALS AND METHODS

Sampling, Plate Counts, and DNA Isolation

Leaf and berry samples from grape (*Vitis vinifera*, 'Chardonnay') were collected from a vineyard near Clarksburg, California, on September 28, 2009. In total, 18 leaf and 3 berry samples were obtained from 3 different locations (A, B, and C) in a single row approximately 30 m apart from each other. The row's orientation was east-west, and at each of the three locations A, B, and C, two leaf samples were picked, one from the north (N) and one from the south side (S) of the row. One leaf sample consisted of leaves 3, 4, 5, and 6 that were pooled from a single shoot (leaves were counted from the shoot tip) and transferred to a plastic bag. The berry samples were taken from the North side at location A. Each berry sample consisted of a single cluster transferred to a plastic bag. Leaf (n=18) and berry (n=3) samples were transported on ice to the lab and processed the same day using protocols that have been established for lettuce (Rastogi et al., 2010). In short, leaf and berry samples were washed by gentle swirling in wash buffer (20 mM Tris–HCl, 10 mM EDTA, and 0.024% Triton), and aliquots were spread on 0.1x Tryptic Soy Agar (TSA) and King's B (KB) plates for counts of colony-forming units (CFUs). The remaining wash solution was centrifuged and DNA was collected from the resulting pellet (representing the grape leaf and berry microbiota), using a PowerSoil DNA Isolation Kit (MO-BIO Laboratories Inc., Carlsbad CA). DNA from 10 randomly selected samples (8 leaves and 2 berries) was sent to the Core for Applied Genomics and Ecology (CAGE) at the University of Nebraska Lincoln for further processing and analysis as described below.

DNA Amplification and Pyrosequencing

DNA from the ten selected samples was used in a PCR with primers PYRO799f (see below) and 1492r (Chelius and Triplett 2009) to amplify bacterial 16S rRNA gene sequences. PYRO799f (5'-ccatctcatccctgcgtgtctccgactcagnnnnnnnnnnAACMGGATTAG ATACCCKG-3') is a derivative of 799f (Rastogi et al., 2010) containing a 16S rRNA gene conserved region (AACMGGATTAGATACCCKG), a unique barcode (nnnnnnnnnn) and a binding site for the pyrosequencing primer (ccatctcatccctgcgtgtctccgactcag). Each PCR reaction was carried out in a 50-µl reaction volume containing 50-100 ng of template, 25 pmoles each of PYRO799f and 1492r, 0.1 uM of each dNTP, 1x Ex Taq PCR buffer (Takara Bio Inc, Mountainview CA), and 1.5

units of high fidelity TaKaRa Ex Taq enzyme (Takara Bio Inc). PCR conditions were as follows: denaturation at 95°C for 5 min, 30 cycles at 95°C for 45 s, 55°C for 45 s, and 72°C for 2 min, followed by a 10-min elongation step at 72°C. PCR reactions were run on a 1% agarose gel and bacterial amplicons with the expected size of 0.7 kb were recovered using a Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA). Pyrosequencing was performed on these amplicons using standard Titanium chemistry. Sequences were parsed through the custom length and quality filters at CAGE and binned by barcode. In order to minimize the effects of sequencing errors, reads that were of atypical length (<200 or >650 bp) or had ≥1 nucleotide mismatch in the primer or barcode sequence were excluded from the analysis. Only reads with an average quality threshold value of ≥20 were considered to be qualified for further analysis.

Analysis of the Pyrosequence Data

From each data set representing a single sample, 1,600 DNA sequences were picked randomly as follows. After opening the original FASTA sequence file of each sample in BioEdit (Hall, 1999), all sequences were selected and all sequence titles were copied and pasted into a text file. This text file was imported into Microsoft Excel, such that all sequence titles were listed in Column A. In column B, the function =RAND() provided each sequence title with a random fraction between 0 and 1. Columns A and B were both selected and then sorted on Column B, after which the top 1,600 sequence titles were selected, copied and pasted into a new text file. This text file was used as the SequenceID file in the FASTA SEQUENCE SELECTION module at the Ribosomal Database Project's (RDP's) Pyrosequencing Pipeline (http://pyro.cme.msu.edu), along with the original FASTA sequence file, to generate a new FASTA file with 1,600 sequences randomly picked from the original sequence file. In the Edit Mode of BioEdit, all sequences were trimmed at the 3' end to a length of 280 bp. These 'FASTA_X' files were analyzed individually, or combined using BioEdit into a single FASTA file ('FASTA_all') representing 16,000 280-bp sequences from 10 samples. 'FASTA_all' or individual 'FASTA_X' files were used as input into RDP Classifier (Wang et al., 2007). Using an 80% confidence threshold, the resulting hierarchy was downloaded as a text file and imported into Excel for quantification of the contribution of indidivual taxa (phylum, class, order, family, or genus) to the total population.

For cluster analysis of the DNA pyrosequence data, FAST_all, FASTA_X, or other FASTA files were uploaded in RDP's Aligner tool. The resulting alignment file was used as input in RDP's Complete Linkage Clustering tool. To find representative sequences within a FASTA file, the RDP module Dereplicate was used. The Basic Local Alignment Search Tool at the PseudomonasDB website (http://www.uib.es/microbiologiaBD/blast/blast.html) was used to assign species names to *Pseudomonas* sequences recovered from leaf and berry sequences.

RESULTS AND DISCUSSION

Culturable Populations of Bacteria on Grape Leaves and Berries

The population sizes of culturable bacteria on grape leaves and berries, as determined by counting CFUs on 0.1x TSA plates, were log-normally distributed (Fig. 1). Leaves collected from the South side of the row contained fewer culturable bacteria (average $10^{5.5}$ per sample) than those on the North side (average $10^{6.0}$ per sample). Variation in population sizes was greater on leaves from the South side (CV=7.9%) than on those from the North side of the row (CV=2.7%). On berries, bacterial population sizes reached an average of $10^{5.4}$ per cluster, but this estimate was based on a smaller sample size (n=3). The numbers of bacteria collected from leaves and berries which were able to form colonies on KB were very similar to those on 0.1x TSA (Fig. 2).

Sequence Analysis of Leaf and Berry 16S rRNA Gene Amplicons

Microbial DNA extracted from 10 randomly selected samples (8 leaves and 2 berries) was used in a PCR reaction to amplify bacterial 16S rRNA gene sequences using universal primers PYRO799F and 1492r (see Materials and Methods). In total, 37,474 high-quality sequences were obtained for the 10 samples combined. The number of reads per sample varied between 1,726 and 6,343, with an average of 3,747±1,920. Average read length was 465±70 base pairs.

To get an overall impression of the bacterial diversity associated with field-grown grape leaves and berries, the 10 data sets were normalized to 1,600 randomly picked sequences from each and pooled into one single data set of 16,000 sequences. Sequences were then trimmed to 280 bp (which covers variable regions V5 and V6 of the 16S rRNA gene) and run through RDP's Classifier. The results of the analysis are summarized in Figure 3. The vast majority of sequences (99.5%) were assigned by Classifier to the domain Bacteria. One percent of all sequences was classified as Cyanobacteria, but in fact represented *Vitis vinifera* chloroplast 16S rRNA gene sequences. We also identified Archaeal sequences (0.3%) which could be assigned to the phyla Crenarchaeota and Euryarchaeota. The occurrence of Archaea on plant leaf surfaces has been reported previously (Delmotte et al., 2009), but their association with grape leaves and berries is a novel finding that invites further investigation. Sequences that did not get assigned to Bacteria or Archaea (0.2%) included 18S rRNA sequences of *Cladosporium* species, which are common fungal colonizers of grape (Tournas and Katsoudas, 2005; Verginer et al., 2010). Our ability to amplify fungal DNA from leaf washings confirms the effectiveness of our protocol to recover fungi and fungal DNA from grape leaves and berries. This information is of great practical value for future investigations into the diversity of leaf- and berry-associated fungal and yeast populations.

A majority (53.5%) of all bacterial sequences could be assigned to the Proteobacteria (Fig. 3). Other well-represented phyla were the Firmicutes (15.1%), Bacteroidetes (10.1%), and Actinobacteria (8.0%). In total, about half of all sequences could be assigned to one of 297 known genera. Among the latter, the most abundantly represented were *Sphingomonas* (7.7%), *Hymenobacter* (4.6%), *Bacillus* (4.3%), *Pseudomonas* (3.7%), *Skermanella* (2.3%), *Leuconostoc* (1.8%), *Massilia* (1.2%), *Methylobacterium* (1.2%), *Cellvibrio* (1.2%), and *Curtobacterium* (1.0%). Together, these top 10 genera accounted for almost one-third (29%) of all sequences. Representatives of *Arthrobacter*, *Pseudomonas*, and *Massilia* showed a more or less uniform distribution among all samples (Fig. 4). In contrast, almost all *Leuconostoc* sequences were found in a single leaf sample, NB2 (not shown).

Different Bacterial Associations with Grape Leaf and Berry Surfaces

Based on our Classifier data, *Sphingomonas* was the most abundantly represented genus on leaves (9.5% of all sequences in 8 leaf samples), while for berries this was *Pseudomonas* (6.9% of all sequences in 2 berry samples). The former is in agreement with previous reports on the prominent presence of sphingomonads in the phyllosphere (Delmotte et al., 2009). Pseudomonads have been isolated from grape berries previously, but not usually as the most abundant type (De La Torre et al., 1998). Genera that appeared to be overrepresented in the grape samples compared to the leaf samples included *Achromobacter*, *Flavobacterium*, and *Cellvibrio* (Fig. 4). The berry-specific association of these bacterial types has, to the best of our knowledge, not been reported before. Genera that were more abundantly represented in the leaf samples included *Sphingomonas*, *Hymenobacter*, *Methylobacterium* and *Curtobacterium*, and, to a lesser extent, *Bacillus* and *Skermanella* (Fig. 4).

We observed a striking difference between grape and leaf samples in terms of sequences that could not be assigned by Classifier down to the genus level. For example, most of the sequences labeled as Unclassified Bacteria were found almost exclusively in the leaf samples (Fig. 5). On the other hand, Unclassified Proteobacteria and Unclassified γ-Proteobacteria were clearly overrepresented in the grape samples (Fig. 5). We combined

into one file all sequences that were labeled as Unclassified Proteobacteria or Unclassified γ-Gammaproteobacteria, and used RDP's Aligner and Complete Linkage Clustering to assess the diversity within this group of 2,312 sequences. We found that 1,856 (80%) were 97% or more identical to each other, which means that they very likely belong to the same genus, if not same species. In the remainder of the text, we will refer to this genus as Most Abundant Unclassified (γ-)Proteobacterium, or MAUP. Upon rerunning Classifier with all 1,856 MAUP sequences, they were distributed more or less equally between Unclassified Proteobacteria (*n*=1067) and Unclassified γ-Gammaproteobacteria (*n*=789), which is in agreement with the results shown in Figure 5.

Most MAUP sequences (76.6%) were found in one of the two grape samples, with the remainder in leaf samples NC2 (17.0%), NB1 (6.1%), and SC3 (0.3%). Within the GR1 and GR2 grape samples, MAUP accounted for 42 and 47% of all sequences, which is significantly more than the percentage of sequences representing the most abundantly represented known genus in the berry samples, *Pseudomonas* (7 and 6.8% in GR1 and GR2, respectively). We did not assess whether representatives of MAUP were among those that were counted as colony-forming units on 0.1x TSB or KB plates (Fig. 1). This culturability of MAUP will be addressed in future studies, together with several other questions. For example, is MAUP a common colonizer on grape berries of other cultivars, in other vineyards and locations in the world, and at subsequent seasons in the same vineyard? What is the mechanism by which MAUPs are enriched on the berry surface? What is their relationship to other genera that are more abundant on the berry surface, including *Achromobacter*, *Flavobacterium*, and *Cellvibrio* (Fig. 4)? Do MAUPS contribute to pre-or postharvest processes, and if so, how?

To assess whether the sequence data could provide information about bacterial diversity beyond the genus level, we analyzed in more detail the sequences that were assigned by Classifier to one particular genus. For this, we chose *Pseudomonas* because a) with 597 sequences it is was one of the most abundantly represented genera in all samples combined and b) *Pseudomonas* sequences were found in comparable numbers on leaves (377 sequences) and berries (220 sequences). Alignment and clustering of all *Pseudomonas* sequences revealed that 90% of them fell into one of two groups which we refer to here as *Pseudomonas* I (329 sequences) and *Pseudomonas* II (211 sequences). Sequences in each group were 97% or more identical to each other and 3% or more different from all other *Pseudomonas* sequences. Figure 6A shows how sequences of *Pseudomonas* I and *Pseudomonas* II were distributed among individual leaf and berry samples. Clearly, *Pseudomonas* I was more dominant than *Pseudomonas* II in berry samples, while the reverse was true for leaf samples. The one exception to the latter was leaf sample NB1, which featured a *Pseudomonas* I to II ratio of 6.1, compared to 0.1-0.4 for all other leaf samples and 15.8 and 12.9 for the two grape samples GR1 and GR2, respectively.

BLAST analysis of 20 random sequences from *Pseudomonas* II revealed that all were 100% identical to the 16S rRNA gene of *Pseudomonas oleovorans*. This species belongs to the *P. aeruginosa* group and includes members that have methylotrophic properties (Egorov et al., 1976). All of the 20 random *Pseudomonas* II sequences originated from leaf samples, including two from NB1. Methylotrophy is a characteristic that has been associated with bacterial survival on plant leaves (Sy et al., 2005). *P. oleovorans* was among the most abundant culturable bacteria retrieved from citrus leaves (Yang et al., 2001) and has also been found in the phyllosphere of other plants (Waight et al., 2007).

Thirteen of the 20 random sequences from *Pseudomonas* I originated from leaves. Two of these (both from sample NB1) were 100% identical to the 16S rRNA gene from *P. caricapapayae*, ten sequences (all from NB1) were 100 or 99% identical to the 16S rRNA genes from *P. rhizosphaerae* and *P. abietaniphila*, and one (from NC2) was 100% identical to the 16S rRNA genes from the Pseudomonas species *fluorescens*, *corrugata*, *chlororaphis*, *lini*, *congelans*, *tremae*, *kilonensis*, *frederiksbergensis*, *thivervalensis*, *migulae*, *jessenii*, *cedrella*, *veronii*, and *mandelii*. The latter finding indicates that the V5-

V6 regions of the bacterial 16S rRNA gene (which are the regions that our sequenced amplicons encompass) do not always offer sufficient resolution to assign sequences at the species level. The remaining seven random sequences from *Pseudomonas* I all came from one of the two grape samples GR1 and GR2 and were identical to the NC2 sequence.

From this, it appears that within *Pseudomonas* I, certain sequences tended to exclusively associate with leaves or with berries. For example, none of the sequences from leaf sample NB1 were found to belong to the '*P. fluorescens* et al' group, whereas all berry sequences belonged to this group. An interesting case is leaf sample NC2: it harbored many *P. oleovorans* sequences (Fig. 6B), which is typical of leaf samples, but also a berry-specific '*P. fluorescens* et al' sequence. We note that NC2 was the only leaf sample with detectable levels of *Flavobacterium*, which was one of the genera overrepresented on berry samples (Fig. 4). We interpret this observation for leaf sample NC2 to mean that movement of bacteria from berries to leaves might occur in the vineyard. This could have important implications for the spread of microorganisms, including pathogens, within the grapevine canopy.

CONCLUSIONS

With this study, we offer a preview into the diversity of bacteria associated with leaves and berries of *Vitis vinifera*. While our sample size was relatively small and our experimental setup not designed to exhaustively answer specific questions, this project produced a number of interesting leads for continued investigation of the microbiota associated with above-ground parts of the grapevine. Our data represent preliminary evidence for bacterial communities on berries that are very different in composition from those on leaves. This is true not only at the level of individual genera, but also at species levels. The dynamics and mechanisms of this selective enrichment remain to be determined, but we can now start to assess them using the sequence data that was generated in this study, e.g. by development and application of genus/species-specific real-time PCR on DNA samples retrieved from vineyards at different spatial and temporal scales. Our discovery of an abundant unidentified (γ-)Proteobacterium on berries invites questions about the generality and significance of this finding and about the putative role, if any, of this bacterium in the pre- and/or post-harvest phase of grape and wine production. The protocol of DNA isolation and amplification appears to work well in our hands for the recovery of fungi and their DNA from grape leaves and berries, which is an essential step towards obtaining a more complete understanding through culture-independent means of the microbial composition of grape leaf and berry surfaces.

ACKNOWLEDGEMENTS

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Figures

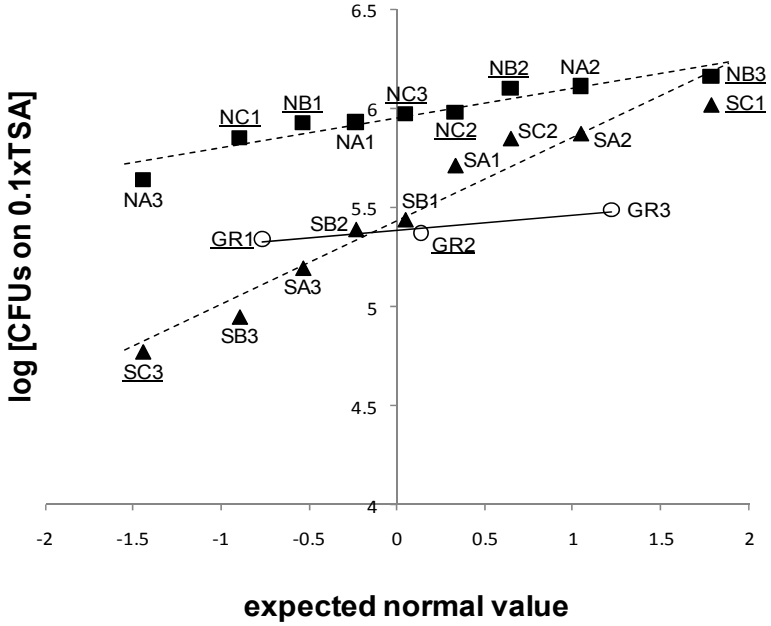

Fig. 1. Bacterial abundances on grape leaves and berries. Shown is a normal probability plot for all of the 18 leaf and 3 berry samples. Abundances are expressed as the logarithm of the number of colony-forming units (CFUs) on 0.1x TSA plates per pool of 4 leaves or per berry cluster. Grape samples are labeled GR1, GR2, and GR3. For the leaf samples, individual data points are labeled according to location along the row (A, B, or C), north- or south-facing origin (N, S), and sample number at that location (1, 2, or 3). For example, NB2 was the second sample that was taken at location B at the north-facing side of the row. Dashed lines and the solid line represent best-fit trends for the leaf and berry data, respectively. The names of those samples for which DNA was extracted from leaf washings for pyrosequence analysis are underlined.

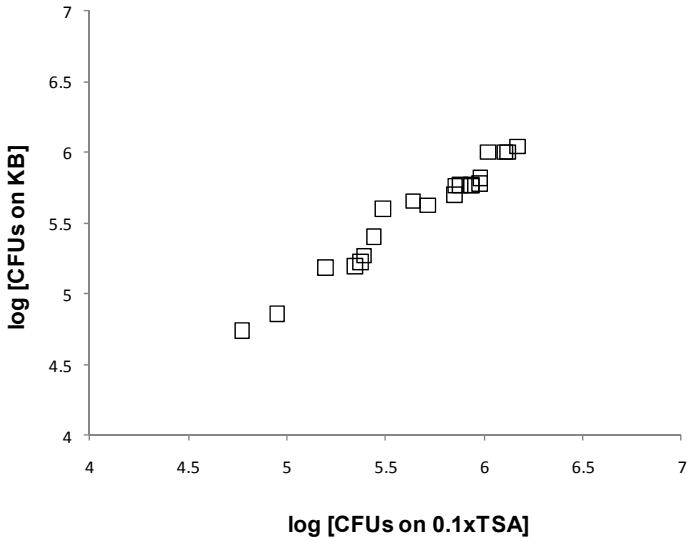

Fig. 2. Comparison of bacterial abundances estimated from colony-forming units (CFUs) on 0.1x TSA versus CFUs on KB. Abundances are expressed as the log CFUs per pool of 4 leaves or per berry cluster. All 18 leaf and 3 berry samples were included to construct this graph.

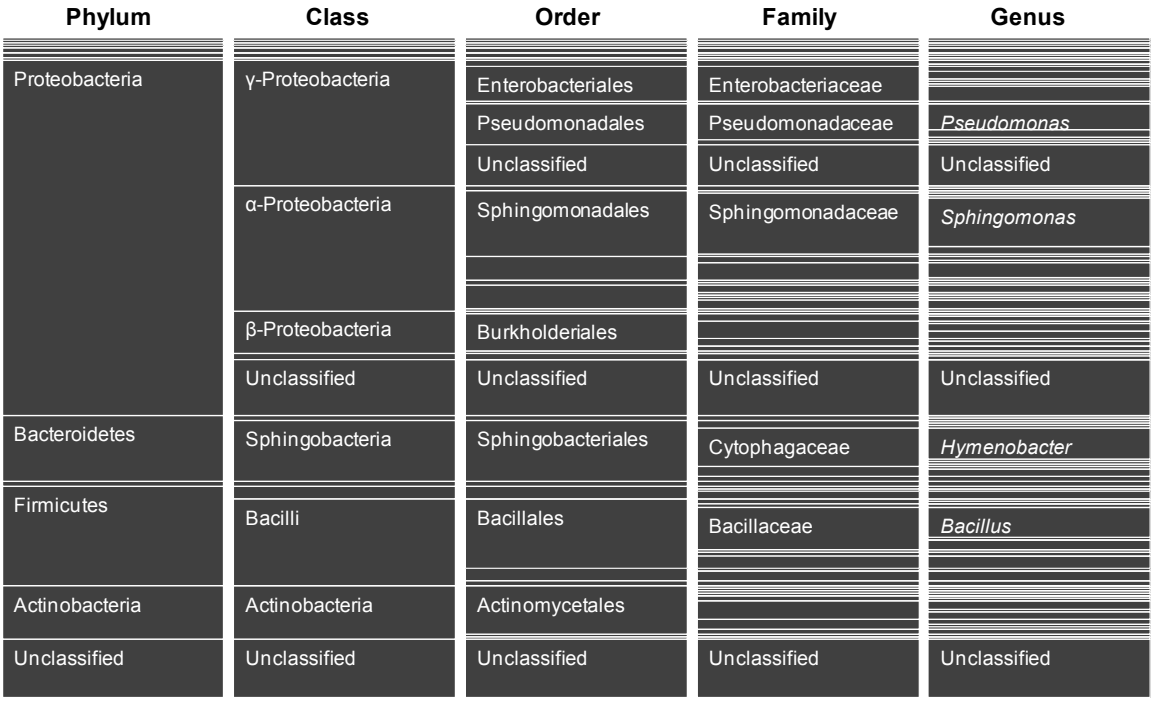

Fig. 3. Bacterial diversity associated with grape leaves and berries. Shown is a summary of the analysis by RDP's Classifier of 16,000 partial 16S rRNA sequences, representing a pool of 10 data sets, one for each of the 8 leaf samples (NB1-3, NC1-3, SC1 and SC3) and 2 berry samples (GR1 and GR2), with 1,600 280-bp sequences each. The five columns represent different taxonomic ranks (Phylum, Class, Order, Family, and Genus), and each grey box within a column represents a distinct taxon within that taxonomic rank. The height of each box corresponds to the percentage of all sequences that were assigned to the taxon that is represented by that box, and the heights of all boxes in a single column add up to 100%. The purpose of the scale bar on the left is meant to facilitate interpretation of the relative height of individual boxes. The boxes of abundantly represented taxa are labeled with the name of those taxa. Interpretation of boxes labeled as 'Unclassified' is context-dependent. For example, the box labeled 'Unclassified' at the bottom of the fifth column represents sequences that could not be assigned by Classifier to any known bacterial Genus, Family, Order, Class, or Phylum, whereas the first 'Unclassified' box in the same column represents Unclassified γ-Proteobacteria, i.e. these sequences could be assigned to the Class γ-Proteobacteria, but not to any Order, Family or Genus within that Class.

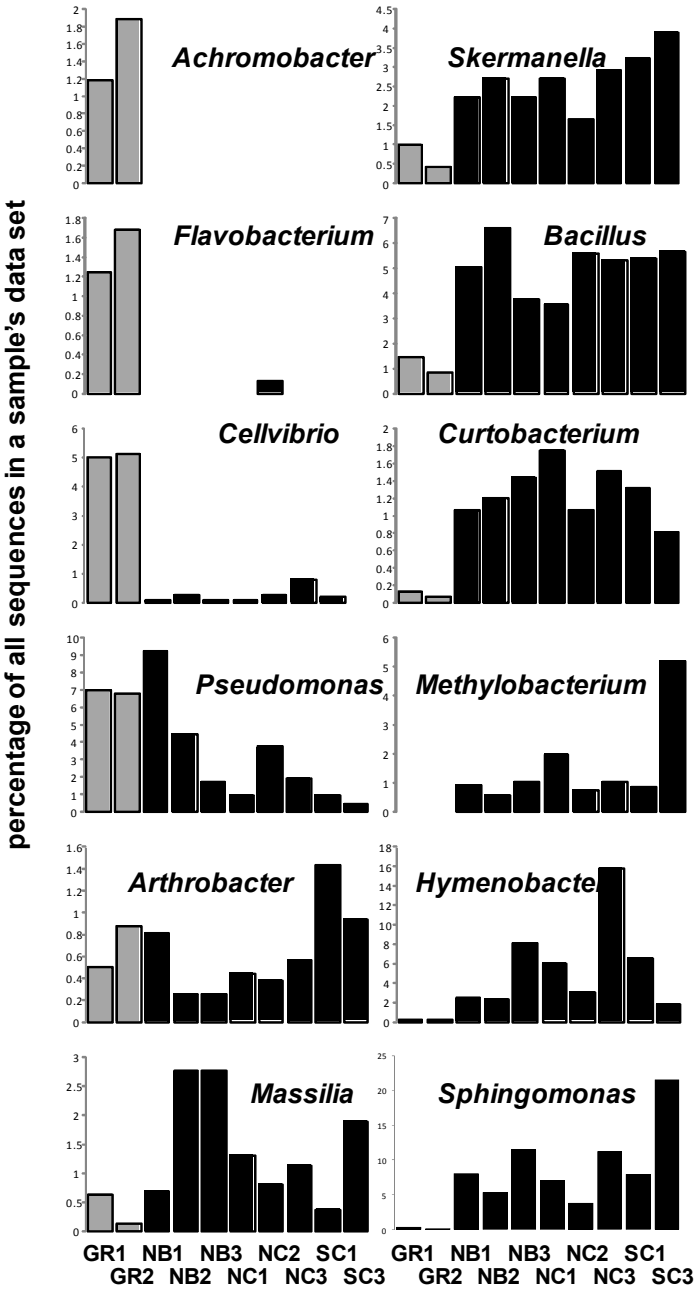

Fig. 4. Comparison of the relative abundance of representatives from selected Classifier-based genera in the bacterial populations associated with grape leaves and berries. For each of the samples GR1, GR2, NB1-3, NC1-3, SC1 and SC3, the relative abundance of twelve bacterial genera is presented on the Y-axis as the percentage of all sequences in the sample data set. Note the difference in scale between the Y-axes. Bars corresponding to grape samples are colored grey, while those of leaf samples are black.

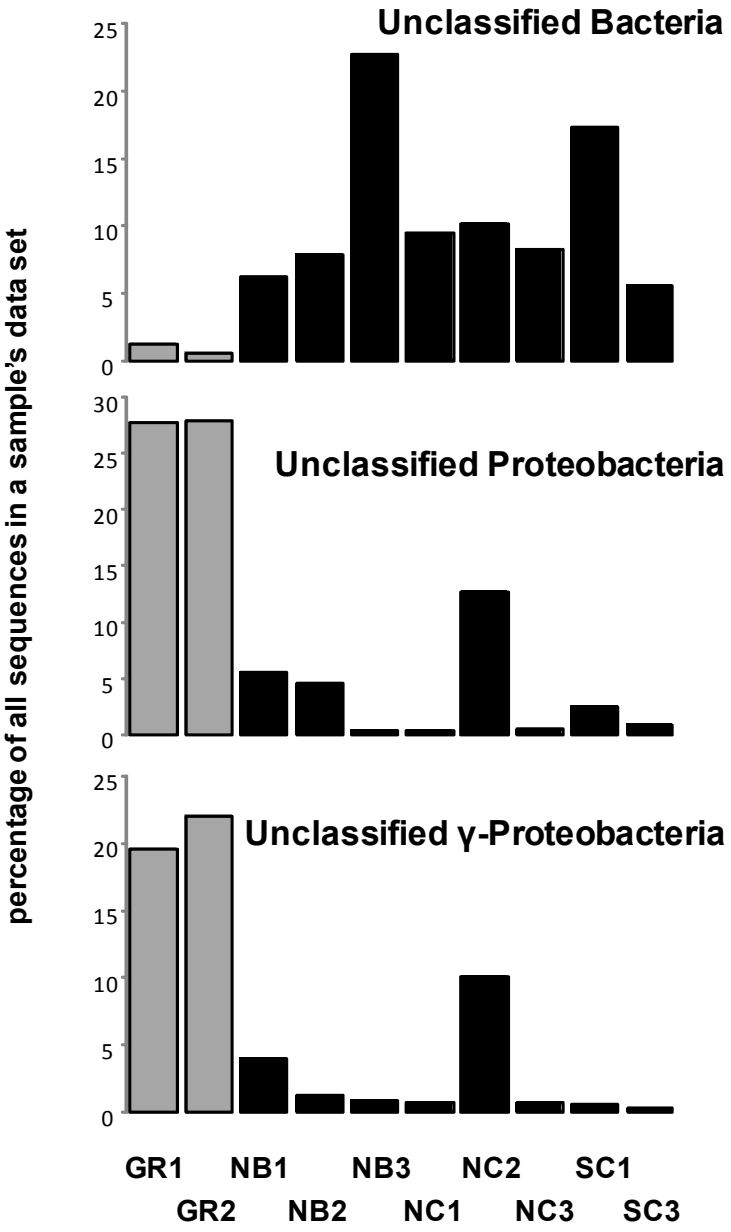

Fig. 5. Comparison of the relative abundance of leaf and berry sequences assigned by Classifier to 'Unclassified Bacteria', 'Unclassified Proteobacteria', and 'Unclassified γ-Proteobacteria'. For each of the samples GR1, GR2, NB1-3, NC1-3, SC1 and SC3, the relative abundance of these sequences is presented on the Y-axis as the percentage of all sequences in the sample data set. Note the difference in scale between the Y-axes. Bars corresponding to grape samples are colored grey, while those of leaf samples are black.

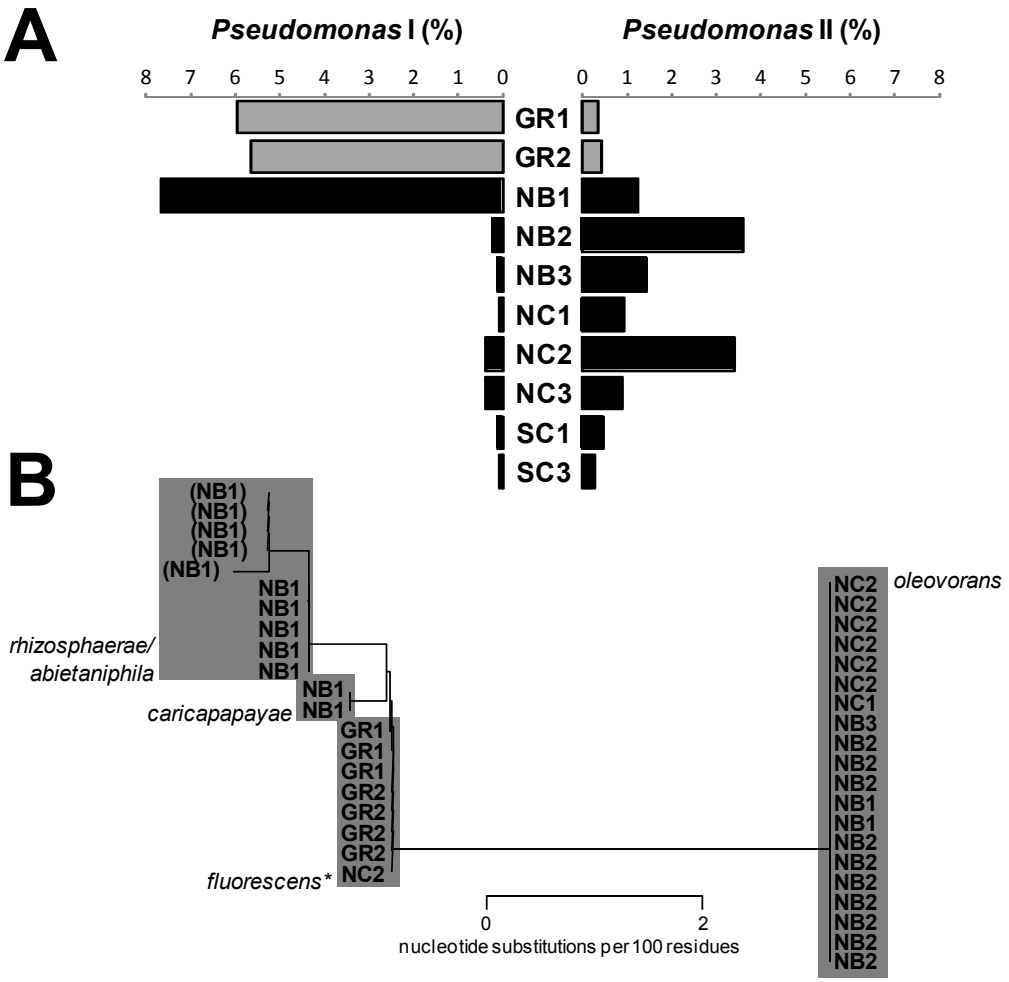

Fig. 6. Differential distribution of *Pseudomonas* species among grape leaves and berries. (A) In this histogram, we show for each of the 10 samples the percentage of 16S rRNA gene sequences which were categorized as belonging to *Pseudomonas* I or *Pseudomonas* II, the two most abundant clusters of sequences with 97% or more identity among all *Pseudomonas* sequences. Bars corresponding to grape samples are colored grey, while those of leaf samples are black. (B) Tree showing the sequence similarity between 20 representatives taken at random from *Pseudomonas* I and 20 from *Pseudomonas* II. Tree is based on the alignment of these sequences. Each sequence is labeled with the name of the sample it came from and falls into one of four groups on the tree: 100% identical to *P. oleovorans*, 100% (99%) identical to *P. rhizosphaerae/abietaniphila*, 100% identical to *P. caricapapayae*, and 100% identical to *P. fluorescens**. The latter category consists of the species *fluorescens*, *corrugata*, *chlororaphis*, *lini*, *congelans*, *tremae*, *kilonensis*, *frederiksbergensis*, *thivervalensis*, *migulae*, *jessenii*, *cedrella*, *veronii*, and *mandelii*. All *oleovorans* sequences originated from *Pseudomonas* II, all other sequences from *Pseudomonas* I.