Culturable bacterial microflora associated with nectarine fruit and their potential for control of brown rot

Wojciech J. Janisiewicz and Jeffrey S. Buyer

Abstract: Microflora of fruit surfaces have been the best source of antagonists against fungi causing postharvest decay of fruit. However, there is little information on microflora colonizing surfaces of fruits other than grape, apple, and citrus. We characterized bacterial microflora on nectarine fruit surfaces from the early stage of development until harvest. Identification of bacterial strains was made using MIDI (fatty acid methyl ester analysis) and Biolog systems. Biolog identified 35% and MIDI 53% of the strains. Thus results from MIDI were used to determine the frequency of occurrence of genera and species. The most frequently occurring genera were Curtobacterium (21.31%), followed by Pseudomonas (19.99%), Microbacterium (13.57%), Clavibacter (9.69%), Pantoea (6.59%), and Enterobacter (4.26%). The frequency of isolations of some bacteria — for example, the major pseudomonads (Pseudomonas syringae, Pseudomonas putida, and Pseudomonas savastanoi) or Pantoea agglomerans — tended to decline as fruit developed. As Pseudomonas declined, Curtobacterium became more dominant. Time of isolation was a significant factor in the frequency of occurrence of different bacteria, indicating succession of the genera. Throughput screening of the bacterial strains against Monilinia fructicola on nectarine fruit resulted in the detection of strains able to control brown rot. The 10 best-performing antagonistic strains were subjected to secondary screening. Four strains reduced decay severity by more than 50% (51.7%–91.4% reduction) at the high pathogen inoculum concentration of 105 conidia/mL.

Key words: biological control, carposphere microflora, resident microflora, stone fruits, postharvest biocontrol.

Introduction

Fruit has a moist, nutrient-rich environment in which resistance to disease decreases as maturation progresses. Fruit surfaces also provide a very good food base for epiphytic microorganisms, both saprophytic and parasitic. In addition to nutrients originating from regular plant leakage, fruit surfaces may contain outside deposits of pollen, organic debris, or honeydew, all of which supply significant amounts of nutrients for epiphytic microorganisms (Blakeman 1985).

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Changes in the nutritional status of fruit as the season progresses can cause changes in the microbial composition on the fruit surface (Williams et al. 1956; Davenport 1976a, 1976b; Singh and Kainsa 1983; Wadia et al. 1983). Fruit pH and the abundance of carbon- and nitrogen-containing compounds (mainly sugars and amino acids) may also determine which group of organisms (bacteria or fungi) predominates (Blakeman 1985). Therefore, it is not surprising that when other environmental factors such as temperature and moisture are favorable, microorganisms may multiply rapidly on the fruit surface.

In the biocontrol of postharvest diseases of fruits, antagonists that are part of the resident microflora of fruit may have certain advantages over antagonists originating from casually deposited microflora and those residing mainly in the soil (Janisiewicz 1987; Chand-Goyal and Spotts 1996; Adikaram and Karunaratne 1998; Droby et al. 1999; Janisiewicz and Korsten 2002). However, the resident microflora of fruit are not well studied, and we know relatively little about the microbial ecology of fruit surfaces. Most of this research has been conducted on grape and apple, often in relation to vinification and apple cider production (Clark et al. 1954; Williams et al. 1956; Davenport 1976a, 1976b; Wadia et al. 1983; Beech 1993) and more recently in relation to bi-
BIOLOG ML 4.2 system (Biolog Inc., Hayward, Calif.) and by fatty acid methyl ester analysis using the microbial identification system (MIS) (MIDI Inc., Newark, Del.). BIOLOG characterization was conducted using GN2 and GP plates for gram-negative and gram-positive bacteria, respectively. BIOLOG data from individual and combined years were subjected to BIOLOG cluster analysis using the ML 4.2 system, and dendrograms expressing units of taxonomic distance were generated (see dendrograms at http://www.ars.usda.gov/pandp/docs.htm?docid=17505). The MIS consisted of an Agilent 6890 GC (Agilent, Wilmington, Del.) and Sherlock software (MIDI). Isolates were grown on trypticase soy broth agar at 28 °C. Cells were harvested, and fatty acid methyl esters were prepared according to the standard MIS protocol. The RTSBA6 method and library were used to perform quantitative fatty acid analysis, calculate the percent area of each fatty acid, and those data were used to classify the isolates. An isolate with a similarity index of 0.2 or greater was considered to be valid at the genus level, while a similarity index of 0.6 or greater was required for an isolate to be considered valid to the species level.

**Preparation of pathogen and bacterial inocula**

The pathogen *Monilinia fructicola* (isolate M17) was isolated from a decayed peach and maintained on peach agar medium under constant light at 22 °C. The peach agar medium contained, per litre, 900 g of canned peach halves in heavy syrup washed 3 times in sterile distilled water and blended in a blender and 22 g agar. Conidia were collected from 10- to 14-day-old sporulating cultures with a vacuum spore collector, suspended in 0.05% Tween 20, sonicated, vortexed, and adjusted with water to a concentration of 10^8 conidia/mL using a hemocytometer. Then, two 10-fold dilutions were made to obtain 2 additional concentrations of 10^4 and 10^3 conidia/mL, which were also used for fruit inoculation.

Bacterial strains were activated from stored stock cultures by transferring them to plates containing NYDA medium. After growing to a sizable culture, they were transferred to plates containing NYDA medium. The pathogen *Monilinia fructicola* (isolate M17) was isolated from a decayed peach and maintained on peach agar medium under constant light at 22 °C. The peach agar medium contained, per litre, 900 g of canned peach halves in heavy syrup washed 3 times in sterile distilled water and blended in a blender and 22 g agar. Conidia were collected from 10- to 14-day-old sporulating cultures with a vacuum spore collector, suspended in 0.05% Tween 20, sonicated, vortexed, and adjusted with water to a concentration of 10^8 conidia/mL using a hemocytometer. Then, two 10-fold dilutions were made to obtain 2 additional concentrations of 10^4 and 10^3 conidia/mL, which were also used for fruit inoculation.

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**Table 2. Abundance of bacterial genera and species isolated from nectarine fruit in 2006 and 2007.**

<table>
<thead>
<tr>
<th>Genus or species</th>
<th>Frequency of occurrence (%) at sampling time T*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
</tr>
<tr>
<td><strong>Burkholderia spp.</strong></td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Chryseomonas luteola</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Clavibacter michiganensis</strong></td>
<td>2.58</td>
</tr>
<tr>
<td><strong>Curvobacter flaccumfaciens</strong></td>
<td>3.23</td>
</tr>
<tr>
<td><strong>Curvobacter pusillum</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Enterobacter intermedius</strong></td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Erwinia sp.</strong></td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Ewingella americana</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Glacoxobacter asaitii, G. cerinus, G. oxydans</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Klebsiella pyrocrensens</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Kocuria kristinae</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Lysobacter sp.</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Microbacterium barkeri</strong></td>
<td>3.23</td>
</tr>
<tr>
<td><strong>Microbacterium lactuca</strong></td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Microbacterium laevaniformans</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Morganella morganii</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Nesterenkonia halobia</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Novosphingobium</strong></td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Pantothea agglomerans</strong></td>
<td>7.10</td>
</tr>
<tr>
<td><strong>Paracoccus sp.</strong></td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Pedobacter heparinus</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Pseudomonas fluorescens</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Pseudomonas putida</strong></td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Pseudomonas savastanoi</strong></td>
<td>1.94</td>
</tr>
<tr>
<td><strong>Pseudomonas syringae</strong></td>
<td>7.10</td>
</tr>
<tr>
<td><strong>Raoultella terrigena</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Rothia spp.</strong></td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Salmonella enterica serovar Typhimurium</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Serratia grimesii</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Sphingomonas yanoikuyae</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Staphylococcus sp.</strong></td>
<td>0.39</td>
</tr>
<tr>
<td><strong>Xanthobacter flavus</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Xanthomonas spp.</strong></td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Yersinia frederiksenii</strong></td>
<td>0.00</td>
</tr>
</tbody>
</table>

*T1 = 6 weeks before harvest (WBH); T2 = 4 WBH; T3 = 2 WBH; T4 = harvest.

*Identified only to genus level at the MIDI cutoff rate of 0.6.
again to NYDA medium, grown overnight, and the bacterial suspensions were prepared by suspending 3 full transfer loops of the culture in 5 mL of sterile tap water for throughput screening against *Monilinia fructicola* on nectarines. For secondary tests, concentrations of the 10 best-performing bacteria were adjusted with a spectrophotometer to 50% transmittance at 420 nm.

**Biological control tests**

Throughput screening of the bacterial strains for control of brown rot was conducted on nectarine fruit. The fruits were washed, randomized, placed on fruit pack trays in plastic boxes, wounded in the middle with a cylindrical tool to produce a cut 3 mm in diameter and 3 mm deep, and the cut tissue was removed. A 25 μL aliquot of the aqueous bacterial suspension was placed in each wound, and after approximately 1.5 h the wounds were inoculated with 25 μL of a conidial suspension of *Monilinia fructicola* at concentrations of 10^5, 10^6, or 10^7 conidia/mL. The fruit were incubated at 24 °C for 5 days, and the lesion size was measured perpendicular to the stem axis. Each isolate was tested on 9 fruits, 3 fruits for each concentration of the pathogen. The 10 best-performing isolates were selected for a secondary test. The methods used in this test were similar to the primary throughput screening, except that the number of fruit per treatment was increased to 9.

**Statistical analysis**

ANOVA and MANOVA were performed using the general linear model (proc GLM in SAS version 9.1.3; SAS Institute Inc. 2004), with year, sampling time, and sample (tree) as the main effects and the percent area of each fatty acid as variables. To test the hypothesis that sampling time affected community composition, we used MANOVA, with sampling time as the main effect and the percent area of each fatty acid as variables. This MANOVA generated linear combinations of fatty acids that best separated group means by sampling time, and these linear combinations were graphed as canonical variates (Seber 1984; Buyer et al. 1999).

We also tested the hypothesis that community composition, as determined by the species and genus names assigned by the MIS to each isolate, was affected by sampling time. Species abundance tables (species or genus vs. sampling time) were constructed and statistically tested using proc FREQ (SAS version 9.1.3). Fisher’s exact test was conducted using Monte Carlo estimation.

**Results**

MIDI identified 89% of the strains to the genus level (0.2 cutoff) and 53% to the species level (cutoff 0.6), while Biolog identified only 35% strains to the species level. Biolog identified 18 different genera, and MIDI identified 28 genera. The identifications by these 2 systems often differ, and we selected MIDI identification for the analysis of the abundance of species. The main genera identified by MIDI were *Curtobacterium* (21.31%), followed by *Pseudomonas* (19.99%), *Microbacterium* (13.57%), *Clavibacter* (9.69%), *Pantoea* (6.59%), and *Enterobacter* (4.26%). All these genera occurred at all isolation times when results from both years were combined (Table 1). All strains among these genera were identified to species (Table 2). There were 7 genera with no identification at the species level. However, lowering the threshold for the similarity indices index from 0.6 to 0.2 resulted in all these strains being identified to the species level, including *Burkholderia andropogonis*, *Burkholderia cenocepacia*, *Burkholderia pyrocinia*, *Erwinia amylovora*, *Lysozyme enzyme*, *Novosphingobium auromaticivorans*, *Novosphingobium capsulatum*, *Paracoccus denitrificans*, *Rothia dentocariosa*, and *Staphylococcus aureus*. None of these genera exceeded 2% of the total occurrence.
Fig. 2. An example of the screening test of bacterial strains against *Monilinia fructicola*, which causes brown rot of nectarine. Wounded fruits were inoculated with a bacterial strain suspension, then, within the next 2 hours, with a conidial suspension of the pathogen at 3 concentrations (P1 = 10^3 conidia/mL; P2 = 10^4 conidia/mL; P3 = 10^5 conidia/mL), incubated for 5 days at 24 °C, and then the diameter of the decay lesion developing around the wound was measured. Strain Ne7T4-S5I7 is *Serratia grimesii*, one of the best-performing antagonists.

![Graph showing lesion diameter (mm) vs. bacterial strain concentration](image)

As fruit developed, the abundance of the 3 most abundant pseudomonads, *Pseudomonas syringae*, *Pseudomonas putida*, and *Pseudomonas sevastanoi*, tended to decline. This tendency was also observed for *Pantoea agglomerans*. Other genera, such as *Burkholderia*, *Erwinia*, *Novosphingobium*, *Paracoccus*, *Salmonella*, and *Staphylococcus*, were only observed during the first 2 sampling periods, but were never dominant. As *Pseudomonas* declined, other genera, particularly *Curtobacterium*, became more prevalent. Several genera, including *Ewingella* and *Morganella*, were only observed during the last sampling period, while *Gluonobacter* was primarily observed during the last sampling period.

In MANOVA, the overall fatty acid composition of all isolates was significantly affected by year (*P* < 0.0001) and by sampling time (*P* < 0.0001) but not by sample (*P* = 0.06, Wilks’ *λ*). When run separately for each year, neither sampling time nor sample were significant in 2006, but in 2007 both were significant (sampling time, *P* < 0.0001; sample, *P* = 0.0092). The fact that the sampling time in MANOVA was significant when both years were combined (*P* = 0.0001) indicates that the effect of sampling time on fatty acid composition was similar in both years, even though it was statistically significant in only one of those years. The MANOVA results are graphed in Fig. 1A, using sampling time as the main effect with data from both years combined. Canonical variate 1 explained 51% of the variance and separated sampling times 2 and 3, with 1 and 4 in the middle. Canonical variate 2 explained 27% of the variance and separated sampling times 2 and 3, with 1 and 4 in the middle. MANOVA generated the linear combination of fatty acids that best separated the samples by sampling time. This linear combination would not necessarily have any taxonomic significance, so we plotted samples averaged by genus (Fig. 1B), using the same canonical variates as previously mentioned. There is considerable separation of the different genera, indicating that the MANOVA did partially separate the samples by taxonomy and also that there was some difference in the genera found at the different sampling times.

The effect of time on species and genus distribution was also tested using frequency tables. For species distribution, only samples with similarity indices ≥0.6 were included (26 species, 155 isolates). The probability of no association between time and species distribution was calculated to be 9.9 × 10^{-40}. For genus distribution, samples with similarity indices ≥0.2 were included (28 genera, 258 isolates). The probability of no association between time and genus distribution was calculated to be 5.9 × 10^{-50}. The MANOVA and frequency analyses were not completely independent of each other, since both were based on the same fatty acid analysis of isolates. However, the MANOVA used fatty acid composition of the isolates directly, while the frequency analyses used the names assigned to each isolate. Both methods provided strong statistical evidence that sampling time significantly affected community composition.

Throughput screening of the bacterial strains for control of brown rot originating from wound infections resulted in the detection of distinctly effective strains (Fig. 2). The 10 best-performing antagonists were selected for secondary screening. These included *Serratia grimesii*, *Gluconobacter asari*, *Raoultella terrigena*, *Pseudomonas fluorescens* biotype B, *Curtobacterium flaccumfaciens-flaccumfaciens*, *Pseudomonas syringae* pv. *atrofaciens*, *Entero bacter intermedium*, *Microbacterium barkeri*, and 1 unidentified strain. In the secondary test, 4 of the strains reduced decay severity by more than 50% at the pathogen inoculum concentration of 10^5 conidia/mL. The unidentified strain reduced lesions by 91.4%, *Microbacterium barkeri* by 84.6%, *Raoultella terrigena* by 57.7%, and *Serratia grimesii* by 51.7%.
Discussion

Curtobacterium spp., Pseudomonas spp., Microbacterium spp., and Clavibacter sp. constituted over 60% of all genera. Many species in these genera could be considered true residents, as they were consistently isolated from the fruit. Other genera were only observed during the early or late samplings, indicating that community succession occurred. This succession may have been due to climatic factors, such as precipitation and temperature (see weather data in the orchard for 2006 and 2007 at http://www.ars.usda.gov/pandp/docs.htm?docid=17505) or to changes in nutrients available to microorganisms growing on the fruit surface (Blakeman 1985).

Interestingly, some of the best bacterial antagonists against pathogens causing postharvest decays of fruits, such as Pseudomonas syringae or Pantoea agglomerans (Janisiewicz and Marchi 1992; Nunes et al. 2002), occurred on fruit at all 4 isolation times, and G. asaisi, shown to be antagonistic against fungal fruit decaying pathogens as well as foodborne human pathogens (Leverentz et al. 2006), was also isolated multiple times. Burkholderia spp., which include the well-known biocontrol agent B. cenocepacia (e.g., formerly B. cepacia genomovar III, and before that Pseudomonas cepacia) (Janisiewicz and Roitman 1988), are ubiquitous in the environment and were also isolated from the nectarines, but only at the beginning of fruit development. Other plant-associated bacteria consistently isolated from nectarines and that may cause plant diseases but have been shown to be effective biocontrol agents in other systems include Clavibacter michiganensis and Curtobacterium flaccumfaciens (Janisiewicz 1990; Lacava et al. 2005). Curtobacterium flaccumfaciens was the most frequently isolated species at all times except T1, when it was the third most abundant species. It was also one of the 10 best-performing antagonists in primary screening on nectarines. This bacterium can cause diseases on soybean and garden beans (Hall 1994; Hsieh et al. 2004), but it was also reported to be endophytic on citrus, where it reduced citrus variegated chlorosis caused by Xylella fastidiosa subsp. pauca, a disease threatening the US citrus industry (Lacava et al. 2007). Because of the endophytic nature of Curtobacterium flaccumfaciens, it may have potential for controlling brown rot originating from latent infections by Monilinia fructicola on nectarines and other stone fruits. Microbacterium lacticum and Microbacterium barkeri were also consistently isolated from the nectarines throughout fruit development. They can be frequently isolated from milk and dairy products (Cullimore 2000). The high biocontrol potential of Microbacterium barkeri, Raoultella terrigena, and Serratia grimesii and the unidentified bacterium strongly suggests that it may be worthwhile to further explore the commercial potential of these strains for biocontrol of brown rot developing from wounds on stone fruits. The potential of these organisms for postharvest control of brown rot originating from latent infections occurring in the orchard is unknown and awaits development of new methods for evaluating this type of microbial interaction. Nevertheless, our findings further confirm the hypothesis that resident fruit microflora are an excellent source of antagonists for control of postharvest decay on fruits (Janisiewicz 1987). The most effective bacterial antagonists in this study are distinctly different from biocontrol agents reported earlier against brown rot on stone fruits, such as Bacillus subtilis isolated from soil, Pseudomonas syringae isolated from apple leaves, or other Pseudomonas and Bacillus species (Pusey and Wilson 1984; Smilanick et al. 1993; Zhou et al. 1999, 2008).

Several food-borne pathogens, such as Salmonella enterica serovar Typhimurium, Staphylococcus aureus, and Pseudomonas aeruginosa, were also isolated from nectarine fruit, but this occurred only sporadically, and their residential status in colonizing nectarine fruit is questionable.

To our knowledge, this is the first report on the bacterial microflora associated not only with nectarine fruit, but also stone fruit in general. Our procedure was designed to maximize the isolation of culturable resident microflora during fruit development over a period of approximately 7 weeks, a technique that could be explored for biocontrol purposes. This information may also be helpful in developing microbial community based biocontrol of brown rot of nectarines (Janisiewicz and Bors 1995). The establishment of the residential status of the bacterial species, however, is more certain for strains consistently isolated throughout fruit development as compare to those occasionally isolated, a difference that may be the result of succession or incidental deposition that was not eliminated during our isolation procedure.

Acknowledgement

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References


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