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RESEARCH PAPER

# Analysis of the bacterial community in glassy-winged sharpshooter heads

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## Abstract

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, is an important vector of various strains of *Xylella fastidiosa*, which cause disease in a variety of economically important plants. These diseases include citrus variegated chlorosis, oleander leaf scorch and Pierce's Disease of grapevines. Symbiotic control (SC) is a new strategy that uses symbiotic endophytes as biological control agents to antagonize or displace the pathogenic strains of *X. fastidiosa*. Candidate endophytes for use in SC must occupy the xylem of host plants and attach to the pre-cibarium and cibarium of sharpshooter insects in order to have access to the pathogen. The study of the bacterial community of GWSS heads by isolation and denaturing gradient gel electrophoresis (DGGE) revealed the presence of species that may be suitable for use in SC. In addition, the results indicated that two important factors, insect age and choice of host plant, affect the composition of the bacterial community in GWSS heads. The main bacterial genera isolated as colonizers of GWSS heads were identified, using partial 16S rRNA gene sequencing, as *Bacillus*, *Pseudomonas*, *Pedobacter* and *Methylobacterium*, as well as the species *Curtobacterium flaccumfaciens*. DGGE patterns revealed a diversity of endophytic species able to colonize the GWSS head. The main genera isolated in culture were also identified using this technique. Principal component analysis (PCA) from polymerase chain reaction (PCR)-DGGE patterns indicated that the bacteria inhabiting the GWSS head are similar to those found as endophytes inside the host plants, and that insect developmental stage and preferential feeding on one host plant species over another are important factors in determining the composition of the bacterial community in the GWSS head. However, a shift in host plants for a small period of time did not cause changes in the compositions of these communities.

**Key words:** endophyte, glassy-winged sharpshooter (GWSS), *Homalodisca*, Pierce's Disease, symbiotic control, *Xylella fastidiosa.*

## Introduction

The glassy-winged sharpshooter (GWSS), *Homalodisca vitripennis*, is a xylophagous insect that feeds on a wide array of host plants, including many ornamental and crop species (Purcell & Hopkins 1996; Purcell & Saunders 1999). This insect is also one of the main vectors of the *Xylella fastidiosa* strain that is the causative agent of Pierce's Disease of grapevine (PD). One new potential management strategy for PD is the use of symbiotic control (SC). Symbiotic control exploits the interactions among a pathogen-transmitting organism, its bacterial symbionts, and the pathogenic organism

**Figure 1** Petri dishes containing bacterial colonies isolated from glassy-winged sharpshooter (GWSS) heads. The right panel is a magnified view of the left panel.

itself (Beard *et al*. 2002). For an SC-based strategy to be successful, a symbiotic bacterium that occupies a similar niche to that of the pathogen must be found. In the case of PD, this requires finding a culturable symbiont that inhabits the pre-cibarium and cibarium of GWSS, since these areas are also colonized by *X. fastidiosa*. A previous analysis of the microdiversity of GWSS heads identified three bacterial genera that met these requirements: *Chryseomonas*, *Ralstonia* and *Alcaligenes* (Bextine *et al*. 2004). The *Alcaligenes* species were of particular interest because they were isolated from wild GWSS (Kuzina *et al*. 2004). Although several *Alcaligenes* species can colonize GWSS, these species do not easily colonize grapevines (Bextine *et al*. 2005). According to Bextine *et al*. (2005), the degree of colonization by *Alcaligenes xylosoxidans* subsp. *denitrificans* (*Axd*) decreased in the following order: orange (*Citrus sinensis*, "sweet orange") > chrysanthemum (*Chrysanthemum grandiflora* cv. "White Diamond") > periwinkle (*Vinca rosea*) > crepe myrtle (*Lagerstroemia indica*) > grapevine (*Vitis vinifera* cv. Chardonnay). Because *Axd* does not colonize grapevines well, two further steps in an SC-based strategy for the treatment of PD are being pursued. First, a bacterial symbiont that can effectively colonize both the grapevine host and the pre-cibarium and cibarium of GWSS must be identified. Second, the major factors influencing the composition of bacterial communities in this insect should be determined, so that they can be manipulated to increase the levels of the symbiont selected for use.

Recently, Marzorati *et al*. (2006) identified *Bacteroidetes* as a novel symbiont of the disease vector *Scaphoideus titanus*, which transmits "*Candidatus* Phytoplasma vitis" in grapevines. The authors used a combination of molecular techniques and transmission electronic micrographs to determine the location of the symbiont inside the insect. The use of culture-independent procedures, primarily based on the variable regions of ribosomal genes, to study bacterial communities is widespread. One such technique, polymerase chain reaction (PCR)-denaturing gradient gel electrophoresis (DGGE), allows the separation of different sequences over a denaturing gradient in a polyacrylamide matrix (Muyzer *et al*. 1993). Reeson *et al*. (2003) also described the bacterial communities associated with wasps (*Vespula germanica*) by applying DGGE-based methods.

The aims of the present study were to describe the bacterial community inhabiting surface-sterilized GWSS heads using both culture-based and PCR-DGGE techniques, and to clarify the possible influence of two variables, insect developmental stage and the plant species used for feeding, in determining the composition of these bacterial communities.

## Materials and methods

### Insect sampling for diversity description

The GWSS adults used in these experiments were collected in July 2005 from citrus (*Citrus sinensis*) and grapevines (*Vitis vinifera*) at the Agricultural Operations groves at the University of California Riverside (UCR Ag. Ops.).

### Isolation and identification of bacteria

The bacteria in the study were isolated from the surface-sterilized GWSS heads using a diluted tryptic soy broth (TSB) medium (Disco; Difco Laboratories, Detroit, MI, USA) (Lauzon & Miller 2002). The DNA used in the analysis was extracted from pure cultures of the bacterial isolates using the Extract-N-Amp DNA extraction kit (Sigma, St Louis, MO, USA). The 16S gene sequence was amplified from the genomic DNA in a total reaction volume of 25 μL, using the degenerate primers 27F (5′-AGAGTTTGATCM-TGGCTCAG-3′) and 1513R (5′-ACGGYTACCTTGTTA-CGACTT-3′) (Zchori-Fein *et al*. 2004). Most samples amplified under the following conditions: initial melt of 95°C, 5 min; 35 cycles of 1 min at 94°C, 40 s at 55°C and 2.5 min at 72°C; and a final elongation at 72°C for 10 min. After amplification, the 16S DNA was purified using the QiaQuick PCR Purification Kit (Qiagen, Valencia, CA, USA), and sent to the UCR Genomics Institute for sequencing. The sequences were run through BLAST (www.ncbi.nlm.nih.gov/BLAST) to obtain the closest

**Table 1** Genera of bacteria cultured from surface-sterilized heads of glassy-winged sharpshooters, *Homalodisca vitripennis*

| Code | Genus | E-value† |
|---|---|---|
| 1w1 | *Bacillus* | 0.0 |
| 3R2 | *Bacillus* | 0.0 |
| 3R3 | *Bacillus* | 0.0 |
| 3y1 | *Bacillus* | 0.0 |
| 4y1 | *Pedobacter* | 0.0 |
| 4y3 | *Pedobacter* | 8.00E–175 |
| 5w3 | *Micrococcus* | 0.0 |
| G18 P1 | *Methylobacterium* | 0.0 |
| G18 P2 | *Methylobacterium* | 0.0 |
| G18 P7 | *Methylobacterium* | 0.0 |
| G18 T10 | *Cryocola* | 0.0 |
| G18 T8 | *Cryocola* | 0.0 |
| G18 W1 | *Microbacterium* | 0.0 |
| G19 P2 | *Methylobacterium* | 0.0 |
| G19 P3 | *Methylobacterium* | 0.0 |
| G19 P5 | *Methylobacterium* | 0.0 |
| G19 P8 | *Methylobacterium* | 0.0 |
| G20 P1 | *Methylobacterium* | 0.0 |
| G20 P7 | *Methylobacterium* | 0.0 |
| G20 P8 | *Methylobacterium* | 0.0 |
| G3R1 | *Bacillus* | 0.0 |
| G3R10 | *Bacillus* | 0.0 |
| G3R11 | *Bacillus* | 0.0 |
| G3R12 | *Bacillus* | 0.0 |
| G3R2 | *Bacillus* | 0.0 |
| G3R3 | *Bacillus* | 0.0 |
| G3R5 | *Bacillus* | 0.0 |
| G3R6 | *Bacillus* | 0.0 |
| G3R7 | *Bacillus* | 0.0 |
| G3R8 | *Bacillus* | 0.0 |
| G18 T9 | *Cryocola* | 5.00E–148 |
| 4o4 | *Rathayibacter* | 0.0 |
| 4o2 | *Rathayibacter* | 0.0 |
| 4o1 | *Rathayibacter* | 0.0 |

†The E-value is a measure of the probability that any similarities in the two sequences were due to random chance and not to a true genetic relationship; the lower the E-value, the more reliable the match can be.

matches, which are shown in Table 1. The last column contains the E-value for each sample, which is provided by the BLAST program. The E-value is a measure of the probability that the match occurred due to random chance and not to any significant genetic relationship. The lower the E-value, the more reliable the match can be.

## Insect development stage comparison

In addition to the 30 GWSS collected for the diversity description, 30 GWSS nymphs were collected and compared with adult individuals in order to observe any differences in the composition of the bacterial community that colonized the head. This comparison was based on PCR-DGGE fingerprint analysis of 60 GWSS (30 adults and 30 nymphs).

## Feeding experiments

The possible effects of the plant host used for feeding in determining the composition of bacterial communities were evaluated in a set of feeding experiments using two different host plants: sweet orange (*C. sinesis*) and grapevine (*V. vinifera*). All comparisons were based on PCR-DGGE fingerprint analysis.

In all feeding experiments, 30 adult GWSS were used for each treatment. A total of 60 insects was collected from each plant species for experimental setup. Of these insects, 30 were maintained on their original plant host, while the other 30 were transferred to a different species of host plant. All insects were maintained in sock cages and fed on their respective hosts for a period of 2 weeks before analysis. Four groups of insects were maintained: insects collected from and fed on citrus; insects collected from and fed on grapevines; insects collected from citrus and fed on grapevines; and insects collected from grapevines and fed on citrus. After this 2 week period, the bacterial communities present in each set of sharpshooter heads were compared.

## DNA extraction from sharpshooter samples

Sampled sharpshooters infected with *X. fastidiosa* were surface-sterilized before DNA extraction according to the following procedure: a 2 min soak in 75% ethanol, then a 2 min soak in 10% bleach, and finally a 2 min soak in sterile double-distilled water. After surface sterilization, each sharpshooter was transferred to a sterile Petri plate and its head and eyes were removed using a sterile scalpel. The eyes were removed because they contain compounds that inhibit PCR. The remainder of the head, which contained the foregut, was placed in a sterile 1.5 mL microcentrifuge tube with 150 µL of sterile phosphate-buffered saline (PBS). This mixture was macerated using an electric homogenizer (Brinkmann Instruments, Westbury, NY, USA) and sterile plastic pestles. DNA extraction was performed using the DNeasy Tissue Kit (Qiagen).

## PCR-DGGE

Amplification of 16S ribosomal RNA gene fragments (rDNA) from genomic DNA was carried out in 50 µL reaction volumes with 400 nmol/L of the 16S universal primers U968_GC (5′-GC_CGAACGCGAAGAACCTTAC-3′) and R1387 (Heuer & Smalla 1997), performing 30 cycles with an annealing temperature of 56°C. The PCR products were analyzed by electrophoresis on 1% (w/v) agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer.

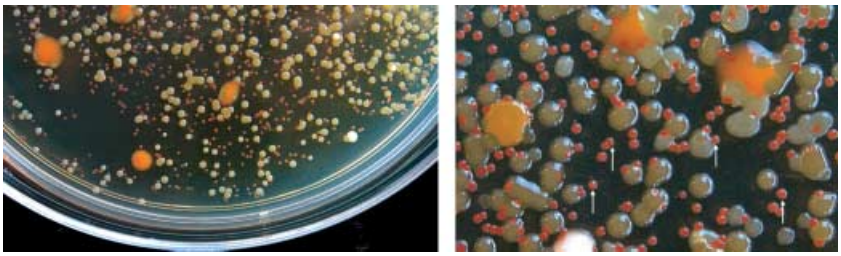

**Figure 2** Petri dishes containing bacterial colonies isolated from glassy-winged sharpshooter (GWSS) heads. The white arrows (right panel, a magnified view of the left panel) indicate *Methylobacterium extorquens* isolated from GWSS heads.

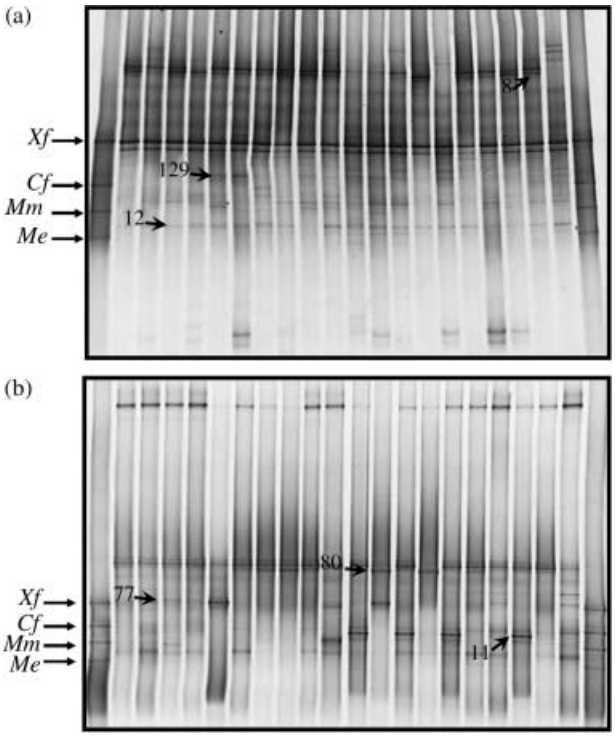


**Figure 3** Denaturing gradient gel electrophoresis (DGGE) fingerprints of the bacterial communities from glassy-winged sharpshooters (GWSS). (a) Young GWSS collected from asymptomatic *Citrus sinensis*. (b) Adult GWSS collected from Pierce's Disease (PD)-infected grapevines and then transferred to and reared on healthy grapevines. *Xf*, *Xylella fastidiosa* (PD strain); *Cf*, *Curtobacterium flaccumfaciens*; *Mm*, *Methylobacterium mesophilicum*; *Me*, *Methylobacterium extorquens*. The numbers 8, 11, 12, 77, 80 and 129 indicate bands in the gels that were identified as *Wolbachia* endosymbionts, *Pseudomonas* spp., unculturable propionibacteria, *Baumannia cicadellinicola*, and *Microbacterium* spp., respectively. These were respresentative of the bacterial genera found in GWSS.

The DGGE was performed as described previously (Muyzer *et al*. 1993) with the Ingeny phorU2 apparatus (Ingeny, Goes, the Netherlands). The amplicons obtained by PCR were loaded onto 6% (w/v) polyacrylamide gels in 0.5× Tris-acetate-EDTA (TAE) buffer. The polyacrylamide gels were made with denaturing gradients ranging from 45 to 65% (where the 100% denaturant contained 7 mol/L urea and 40% formamide). The gels were run for 16 h at 100 V and 60°C, after which they were soaked for 1 h in SYBR Green I nucleic acid stain (1:10 000 dilution; Molecular Probes, Leiden, the Netherlands) and immediately photographed under ultraviolet (UV) light.

### PCR-DGGE data analysis

The GelComparII program (www.applied-maths.com/gelcompar/gelcompar.htm) was used to normalize and convert gel images. Standardizations made by GelComparII were carefully checked manually and corrections were made when necessary. A table containing band positions and their presence or absence in every sample was exported for further analysis. Principal Component Analysis (PCA) was carried out using CANOCO 4.5 software (Microcomputer Power, Ithaca, NY, USA). The table containing band positions and presence/absence data was used as species data.

## Results and discussion

The use of isolation (Figs 1,2) and DGGE (Fig. 3) techniques revealed several genera of bacteria as colonizers of GWSS heads. As identified by 16S sequencing, these included *Bacillus*, *Cryocola*, *Microbacterium*, *Micrococcus* and *Pedobacter*. In addition, *Methylobacterium extorquens*, *Curtobacterium flaccumfaciens*, *Baumannia cicadellinicola* and various *Pseudomonas* and *Wolbachia* species were found (Fig. 3). Of the genera, *Bacillus*, *Pseudomonas*, *Methylobacterium* and *Curtobacterium* have already been described as endophytes that are able to colonize citrus plants. Recently, the work of Araújo *et al*. (2002) strongly indicated interactions among *Methylobacterium* spp., *Curtobacterium flaccumfaciens* and *X. fastidiosa*. These results reinforce the idea that all of these bacteria could interact in the vector insect as well as in the host plant. Furthermore, Lacava *et al*. (2004) suggest that citrus variegated chlorosis (CVC) symptoms in citrus plants could be a result of the interactions among these three species.

Our study of the diversity of bacterial communities associated with GWSS foreguts used culture-dependent methods as well as procedures based on sequence polymorphisms

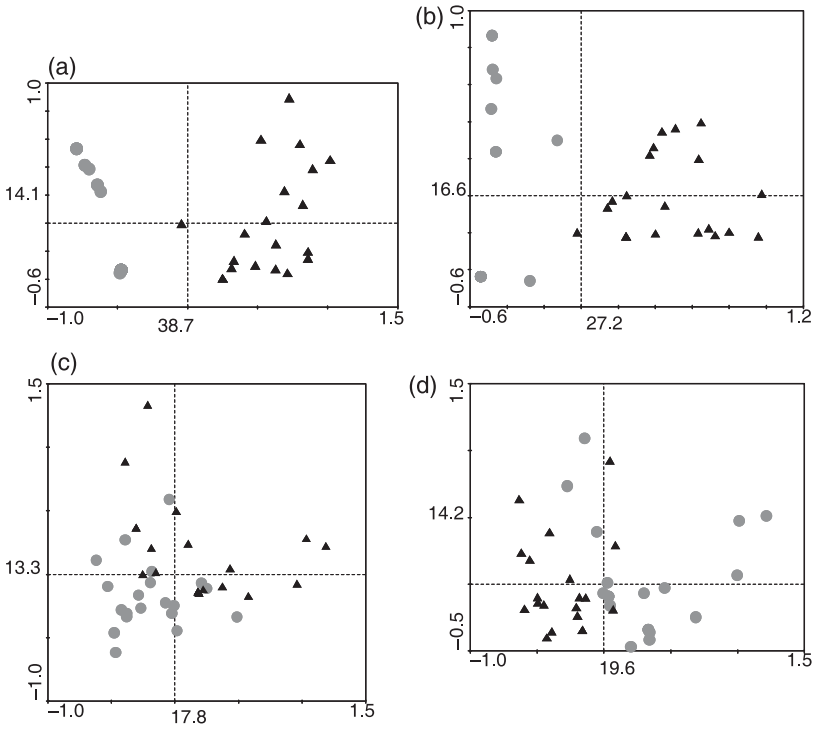


**Figure 4** Principal component analysis (PCA) obtained from denaturing gradient gel electrophoresis (DGGE) patterns of bacterial communities from the foreguts of glassy-winged sharpshooters (GWSS; *Homalodisca vitripennis*) at (a) different stages of development (●, adult; ▲, young), (b) different plant feeding (●, citrus; ▲, grapevine) and (c,d) subjected to new plant feeding (c: ●, citrus–citrus; ▲, citrus–grapevine; and d: ●, grapevine–citrus; ▲, grapevine–grapevine). Every group presented in the graphs is composed of 30 individuals of *H. vitripennis*. In (a), due to high similarity of samples, some samples are overlapped. Values on the axes indicate the percentage of data variance explained in the respective axis.

(DGGE) of the 16S gene using total DNA extracted from GWSS foreguts. The diversity profiles obtained with isolation techniques indicated a low bacterial diversity. However, higher bacterial diversity was observed using PCR-DGGE, a culture-independent method. These preliminary results show that PCR-DGGE is suitable for the analysis of bacterial diversity in GWSS heads. In the future, species such as *Curtobacterium flaccumfaciens* and *Methylobacterium* spp., found in part of the bacterial community in GWSS, could be investigated as potential candidates for use in an SC-based strategy to control the spread of PD.

The composition of bacterial communities is highly dependent on biotic and abiotic factors (Kozdroj & Van Elsas 2000; Marschner & Baumann 2003; Andreote *et al*. 2004; Salles *et al*. 2004). In the case of insects that feed on a variety of species, foregut communities can be highly dynamic, changing over time and also in response to shifts in insect feeding patterns. To account for these factors, PCR-DGGE analysis was performed on young and adult insects, as well as on insects collected from citrus and fed on grapevine (and vice versa). The results of these experiments can be seen in Figure 4.

A comparison between young and adult insects revealed that young insects harbor a different bacterial community than do adult insects (Fig. 4a). These results may be linked to the ability of young and adult sharpshooters to feed on different plant tissues, which would then select for the presence of particular groups of bacteria in their heads.

For adult insects, the plant species from which they were collected was demonstrated to be an important factor in determining the composition of the bacterial community in the head. A comparison of head bacterial communities in GWSS collected from grapevine and in GWSS collected from citrus showed the presence of unique PCR-DGGE fingerprints for each group (Fig. 4b). However, cross-feeding the insects collected from citrus on grapevine (and vice versa) did not cause a dramatic shift in the compositions of the respective bacterial communities (Fig. 4c,d). In these analyses, a clear separation of samples did not occur, and the percentage of variance expressed in the axis was also lower. These results indicate that other factors also influence the composition of these communities.

A comparative analysis of bacterial communities in GWSS nymphs and adults demonstrated that the composition of the bacterial community in the foregut changes over the life of the insect, and the possibility that it is influenced by feeding on separate parts of the plant. In addition, when bacterial communities from insects feeding on different plant species were assessed, major differences were found. Because these differences in feeding had such a major effect on the composition of the foregut bacterial community, this information should be taken into account in future studies involving the use of SC to control the spread of *X. fastidiosa*.

The results presented in this article supply essential information about bacterial species that may be suitable in the use of an SC-based strategy to control the spread of *X. fastidiosa*.

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