

## EXPLORATION FOR FACULTATIVE ENDOSYMBIONTS OF SHARPSHOOTERS

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**Reporting Period:** The results reported here are from work conducted from July 1, 2002 to June 30, 2004.

### ABSTRACT

Glassy-winged sharpshooters (GWSS) were collected in California and several states in the southeastern United States in 2002 and 2003 to search for pathogenic or beneficial endosymbiotic bacteria of these insects. Various tissues were examined for the presence of bacteria by PCR: hemolymph, eggs, and bacteriomes. A subset of hemolymph and egg samples were cloned and sequenced based on unique digest patterns of their extracted 16S rDNA, or analyzed by restriction digest patterns of sample compared to known bacterial DNA. Most cloned sequences were identified as *Baumannia* (one of the primary symbionts of GWSS), and *Wolbachia* (a common secondary symbiont in a majority of insect taxa investigated). In addition, we isolated bacteria that were most closely related (by 16S rDNA sequence) to the following genera: *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas*, *Burkholderia*. All are common bacteria that are found in soil, water, or plant surfaces, and also in insect guts or surfaces.

### INTRODUCTION

We have surveyed populations of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*, for bacterial symbionts that might be exploited to manipulate the biology of this insect vector of *Xylella fastidiosa* (Xf) (Purcell and Feil 2001). Pathogens or other microbial associates of GWSS have not been employed to date as biological control agents or contributors to the control of these pests largely because none are known, although some efforts to discover viruses of GWSS have been made. Although endosymbiotic bacterial associates of leafhoppers are little-understood and unexploited to date, their potential importance is well worth exploring. The first step has been to look for and identify any naturally occurring bacteria in GWSS populations from a wide geographical range.

Of particular interest to us in this study were bacterial associates that are facultative (also referred to as “secondary”), i.e., that occur in some individuals or populations but are not required by their hosts; and that could be introduced into, or augmented in pest populations. We use the term symbiont here in the biological sense of “living together” and do not imply mutual benefit (Douglas 1994). Facultative bacterial associates have been described in a variety of homopterans including leafhoppers (Swezy and Severin 1930, Schwemmler 1974, McCoy et al. 1978, Purcell et al. 1986). The only leafhopper facultative symbiont studied in some depth is BEV, a bacterium that occurs in *Euscelidius variegatus* in France, but apparently not in California (Purcell et al. 1986). Uninfected females of *E. variegatus* inoculated with cultures of BEV transmitted the bacteria transovarially (“vertically”) to their offspring, with resulting deleterious effects (Purcell et al. 1986, Purcell and Suslow 1987). This bacterium could also be transmitted horizontally between leafhoppers feeding on the same plant; hence it could persist in the population in spite of its negative fitness effects.

It is clear from our studies of facultative bacteria in aphids (Chen et al. 2000, Montllor et al. 2002) as well as from the study of BEV, that endosymbiotic associations are complex and have critically important effects, both positive and negative, on the physiology, population biology and vector potential of their hosts. Some of the most extensive studies on the effects of facultative symbionts on insect hosts involve *Wolbachia*, a transovarially transmitted bacterium that occurs in 20-76% of investigated insect species (Weeks et al. 2002) with a range of interesting effects (e.g., Werren 1994, Stouthammer et al. 1999). *Wolbachia* has recently been described from GWSS (Moran et al. 2003), though its effects remain unknown. Although *Wolbachia* has “helped raise the awareness of the potential contribution of endosymbionts...it is important not to discard other alternatives” (Weeks et al. 2002). Our approach was to investigate whether other alternatives existed for GWSS.

## OBJECTIVES

1. Survey glassy-winged sharpshooter and other sharpshooters in California and the southeastern United States for facultative bacterial endosymbionts and determine by DNA sequencing the identity of any bacteria discovered.
2. Depending on type of microorganism and relative frequency in surveyed insects, select candidate symbionts to determine biological effects on GWSS.

## RESULTS

We collected GWSS from various locations in California and in Louisiana and Florida in spring and summer 2002. In June 2003 we collected GWSS from Louisiana, Mississippi, Alabama and Florida. Four other species of sharpshooter were also collected in California in summer 2002 and fall 2003. Some field collected GWSS from selected locations were brought back to the lab and caged together for one to several weeks in order to facilitate exchange of any potentially horizontally transmitted facultative symbionts. In several cases, long-term lab colonies were established from field populations, and could be repeatedly sampled. Laboratory-reared GWSS were also obtained from the California Department of Food and Agriculture rearing facility in Bakersfield, California on several occasions in 2003.

DNA from three types of tissue from sharpshooters collected in 2002 and 2003 were extracted: hemolymph, eggs, and bacteriocytes. Over 400 extractions have been made and analyzed for bacterial DNA. Hemolymph is known to contain bacterial endosymbionts in aphids (e.g., Chen et al. 1996) and leafhoppers (e.g., Purcell et al. 1986) and is a logical place to sample. Approximately 2- 4 uL of hemolymph was removed by puncturing the abdomen with a glass needle, and was then added to 20 uL phosphate buffered saline (PBS) and stored frozen until analysis. After extraction, we amplified the DNA of the 16S ribosomal DNA with “universal” bacterial primers, digested any bacterial DNA with restriction enzymes, and looked for different patterns that might indicate the presence of more than one type of bacteria. A subset of bacterial 16S rDNA was cloned in *E.coli*, reanalyzed with restriction enzymes (e.g., Table 1), and sequenced if deemed appropriate. This procedure was also applied to eggs (dissected from gravid females or removed from leaves after being laid) in which we expected to find any vertically transmitted endosymbionts, such as the primary symbionts, *Baumannia*, but perhaps other symbionts as well.

Forty-five percent (126/281) of hemolymph samples from all localities tested positive for bacterial 16S rDNA by PCR. Twenty-six individuals of another four species of sharpshooters from California were also tested for bacteria in hemolymph, of which five (19%) were positive by PCR. We have not yet analyzed these further. DNA from a total of 25 GWSS tissue samples from 17 individuals was chosen for cloning, and 19 produced multiple transformed *E. coli* colonies with bacterial 16S rDNA inserts. DNA from 45 of these colonies was chosen for sequencing, and others were identified by restriction digest analysis. The most common sequence was identical to that of *Baumannia*, a bacteriome-associated symbiont of the GWSS (Moran et al. 2003) (Table 1). Like other bacteriome inhabitants, *Baumannia* is presumably transovarially transmitted from mother to offspring via hemolymph (Buchner 1965). *Wolbachia*, a commonly found facultative symbiont of many insects, including GWSS (Moran et al. 2003), was also cloned or commonly found facultative symbiont of many insects, including GWSS (Moran et al. 2003), was also cloned or otherwise identified from hemolymph and eggs of California, Florida and Louisiana GWSS. In addition, we surveyed extracted DNA that was positive for 16S rDNA for *Wolbachia* by PCR. *Wolbachia* has been described from GWSS (Moran et al. 2003), but its prevalence and the existence of strain differences has not been documented. We found *Wolbachia* in 10% (8/84) of hemolymph samples and 59% (19/32) of egg samples. These figures are probably conservative, and indicate that *Wolbachia* is a very common bacterium associated with GWSS. *Baumannia* was amplified from 67% (60/89) of hemolymph samples by PCR.

<b>Table 1.</b> Cloned bacterial DNA from GWSS tissue samples. Bau=Baumannia; Wol=Wolbachia, Aci=Acinetobacter; Pseu=Pseudomonas; Burk=Burkholderia.		
Collection location (sample / no. clones sequenced or digested)	GWSS tissue	16s rDNA identity of inserts (by sequencing or restriction digest analysis)
Bakersfield	Hemolymph Eggs	Bau, Wol, Aci Wol, un-id
CDFA	Hemolymph Eggs	Bau, Wol, un-id
Louisiana State Univ	Hemolymph Eggs	Bau, Sten, Pseu Bau
Crestview FL	Hemolymph Eggs	Bau, Wol, Aci, Pseu Bau, Wol
Pearl River LA	Hemolymph Eggs	Bau, Wol, un-id Bau, Wol, Burk

Although *Baumannia* and *Wolbachia* were the most common bacteria found, a few other 16S rDNA of bacteria not previously described from GWSS were also cloned from GWSS samples (Table 1). Some samples are still being analyzed to determine the identity (“un-id” in Table 1) or close relationship of the bacteria represented. Among those isolated were bacteria with identity similar to *Acinetobacter*, *Stenotrophomonas*, *Pseudomonas* and *Burkholderia*. All are aerobic  $\gamma$ -Proteobacteria, and not uncommon as environmental contaminants and nosocomial pathogens (e.g., Towner et al. 1991, Ribbeck et al. 2003). However, *Acinetobacter* and *Stenotrophomonas* have also been isolated from ticks and fleas (Murrell et al. 2003); and *Stenotrophomonas*, among other bacteria, was isolated from the guts of ants, where it was presumed to provide nutrients and to be passed to offspring (Jaffe et al. 2001). *Stenotrophomonas* was also described as an endosymbiont of a fly (Otitidae), which did not develop properly without its complement of bacteria (Wozniak and Hinz 1995). *Burkholderia*, a pseudomonad, was isolated from termite guts (Wertz et al. 2003), and was able to colonize a variety of aquatic invertebrates both externally and internally (McEwen et al. 2001).

We did not detect any bacteria in PBS buffer alone. Bacteria were detected in 4 of 12 buffer samples that were pipetted onto the outside surfaces of 12 different insects. We were only able to clone one of these DNA samples because subsequent PCRs of the other three were negative for 16S DNA. The cloned sample contained 16S DNA similar to that of *Pseudomonas*, *Acinetobacter*, and *Methylobacterium*. It is not yet possible, therefore, to determine whether *Acinetobacter* and *Pseudomonas* cloned from hemolymph samples came from the insect surface, the hemolymph, or both.

## CONCLUSIONS

A wide-ranging search for secondary symbionts of the GWSS did not identify good candidates for studies on biological effects on this insect. Some bacteria we identified were possibly from insect external surfaces. The prevalence of a *Wolbachia* species, and the well-known importance of *Wolbachia* to other insect hosts make it the best candidate to pursue in further studies.

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#### **FUNDING AGENCIES**

Funding for this project was provided by the University of California's Pierce's Disease Grant Program and the University of California, Berkeley College of Natural Resources ARE Institute.