SHARPSHOOTER ASSOCIATED BACTERIA THAT MAY INHIBIT PIERCE'S DISEASE

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INTRODUCTION

Xylella fastidiosa is the etiological agent of Pierce's disease, an important disease of grapes in the United States. This disease limits viticulture in Florida and the rest of the southeastern US. It was observed in the Temecula Valley of California in 1997. Though this disease had been known in southern California (California Vine Disease, Anaheim Disease) since the 1880's, it had not been reported before in the Temecula Valley wine grape area. The appearance of Pierce's disease in Temecula coincided with increased populations of the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata*. Because of the mobility and vector capacity of this insect, Pierce's disease has become a cause for great concern to wine industry in California.

We plan to culture, identify, then select or genetically transform insect-associated bacteria, especially gut bacteria, from glassy-winged sharpshooter to produce substances that inhibit or kill *X. fastidiosa.* We also intend to use bacterial-plasmids derived from gram-negative bacteria and the novel *himar* transposon/transposases-mediated system derived from the *mariner* insect transposon.

OBJECTIVES

1. Identify insect associated bacteria in GWSS and related insects in Southern California or other areas where the vector insects are endemic.
2. Evaluate identified bacteria for the production of antibiotics inhibitory to *Xylella* for potential hazards to plants animals and humans and for their potential for genetic manipulation through techniques for genetic transformation. Those that can be genetically transformed will be investigated for their ability to express transgenes that produce substances that inhibit, attack, destroy, or prevent the transmission of *X. fastidiosa.*
3. Evaluate various peptide antibiotics demonstrating effective inhibition of *X. fastidiosa* that could be candidates for introduction and production in the GWSS-associated bacteria.

RESULTS AND CONCLUSIONS

Methods of collection and isolation of GWSS was simplified. At the beginning of our work on GWSS gut bacteria, the prevailing thoughts were that the propensity for GWSS to promiscuously acquire environmental bacteria was represented as very high— these insects would pick up practically anything. On further study, it was found that this propensity referred to surface contamination, not gut bacteria. That is, a wide range of bacteria of environmental nature could be cultured from the surface of unsterilized GWSS but not necessarily from solely their internal structures. However, the previous studies of GWSS bacterial flora had been performed to detect any bacteria associated with GWSS and were not necessarily performed with an eye to the exclusion of surface contamination. Our investigations of gut bacteria had been done after having taken especial pains not to destroy bacteria in the gut or other internal organs of this insect.

The thought that GWSS indiscriminately picks up environmental bacteria guided our original collections of this insect. Because of concern over casual contamination of collected GWSS, field collections of GWSS were done by trapping the insects by hand directly from vegetation and placing them in clean, unused Ziploc polyethylene baggies. These baggies are quite clean if not sterile. We were working with the concern that GWSS could pick up environmental bacteria from surfaces, particularly that of a sweep net, which could contaminate the gut flora. The time needed for this hypothetical contamination of GWSS gut to happen was completely unknown so we erred on the side of caution in our initial collection procedure. We wanted to collect uncontaminated insects so as to be certain of the origin of the bacteria we isolated from their guts. This method of collection was used for several collection cycles in the Southern California area. However it was extremely laborious and it was very difficult indeed to capture these very active and alert insects in a plastic baggie, sometimes an entire afternoon was used in pursuit of a single GWSS where populations of GWSS were low (vineyards).

Hence, in an effort to determine the necessity of such elaborate and inefficient collection measures, an experimental collection was made in which 10 insects were captured with the baggies and 10 were collected in a sweep net at the same site on University of California, Riverside's campus agricultural research lands. If the contamination of the gut was rapid, we would expect to see differences between the gut flora of the insects collected with the baggies and those with the net. Subsequent microbiological study of both groups of insects revealed no differences in the gut flora. Therefore, subsequent GWSS collections for purposes of obtaining gut bacteria were made with the much more efficient sweep net.

In retrospect, this discovery makes sense. The initial assumption that GWSS picked up environmental bacteria promiscuously was based on previous surveys in which the unsterilized insects were rolled over the surface of a petri dish and the resulting bacterial colonies were then isolated, purified, and identified. In contrast, our studies were performed on insects which were collected in the field, first by grabbing the insects with clean baggies, and then storing them for a short time in clean fresh baggies with a sprig of host plant material as food for the GWSS until further processing. Within two hours of collection, GWSS were removed from the baggies with sterile instruments, then they were cooled to 4 ^{0}C to reduce their mobility. They were then placed with sterile instruments in sterile mineral oil at about 20 ^{0}C and frozen at -80 ^{0}C. The mineral oil sealed in the contents of the gut at both the anus and mouth and prevented any bacteria from subsequently entering the GWSS during storage or transit. For gut bacteria isolation, the frozen insects were removed from the oil, and then exhaustively surface-sterilized in 70% ethanol/3% sodium hypochlorite solution for five minutes. The surface sterilized (and quite dead) GWSS were then transferred to sterile insect Ringer's solution and aseptically dissected to remove the gut. The gut and contents were plated on nutrient agar and incubated for the growth of bacterial colonies. These colonies were purified by restreaking and analyzed through biochemical and nucleic acid analysis to establish the identity of the bacteria. Subsequent to discovery of bacteria in the gut, collections of GWSS were made throughout the year to establish whether these bacteria were always found in GWSS as a part of the normal gut flora. In the case of at least *Alcaligenes xylosoxidans denitrificans*, this appears to be so.

Various bacteria like *Alcaligenes xylosoxidans denitrificans, Chryseomonas luteola, Arthrobacter* sp., *Bacillus* sp. and *Ralstonia* sp.are reported. The most recent analysis of GWSS gut bacteria have included three yet to be fully characterized bacteria. *Ralstonia* was formerly known as *Alcaligene* and it has similar biochemical characteristics to this genus. Further biochemical characterization should allow us to identify it to species. The *Arthrobacter* sp. and *Bacillus* sp. are gram-positive bacteria. Since there is a great deal known about the biology of *Bacillus,* and a number of dependable expression vectors for transformation of this bacteria, this particular bacterium may have promise as a vehicle for production of substances inhibitory to *X. fastidiosa* within either the plant or GWSS, or to actively destroy the GWSS through engineering of this *Bacillus* to produce a toxin detrimental to GWSS. Perhaps some sort of *Bacillus thuringiensis* toxin might be employed in this fashion. *Arthrobacter* species are considerably less understood. The distribution and frequency of occurrence of these bacteria must be further studied to understand how they may be used in a program to control Pierce's disease and/or the GWSS.

The *Alcaligenes* have thus far been found in all the GWSS collections. This suggests a potential symbiotic relationship with GWSS that also needs to be further investigated. Unlike *Alcaligenes xylosoxidans denitrificans, Chryseomonas luteola* has been very slow growing on typical laboratory media and is quite difficult to culture. We have been concentrating on culture and transformation of *Alcaligenes xylosoxidans denitrificans* because of ease of its laboratory culture and the observation that it has been found in all the GWSS so far collected. Additionally, the biochemical characteristics (such as especially good growth in low nutrient conditions) of these GWSS bacterial isolates suggest a plant affiliation or origin for these bacteria. We speculate that the GWSS may be picking up these bacteria from their host plants. As GWSS feeds solely on xylem, the source of these bacteria may very well be plant xylem. Further study is needed to investigate this.