

INSECT-SYMBIOTIC BACTERIA INHIBITORY TO *XYLELLA FASTIDIOSA* IN SHARPSHOOTERS

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INTRODUCTION

The appearance of Pierce's disease vectored by a relative newcomer to California, the glassy-winged sharpshooter, *Homalodisca coagulata*, has lead to the project outlined here. We hope to develop a new strategy to combat the spread of Pierce's disease by delivering anti-disease strategies aimed at neutralizing the disease organism, the bacterium *Xylella fastidiosa*, its production of a form of xanthan gum that clogs the xylem spaces of the plants or disrupts connection of the pathogen to the mouthparts of the vector insect. The use of bacteria associated with insects to disrupt disease transmission is itself a new approach to disease control. The main principle, paratransgenesis, which is the genetic alteration of bacteria carried by insects, was originally designed by Frank Richards to disrupt the transmission of Chagas disease by Triatomine bugs. The possible use of paratransgenesis for preventing Pierce's disease was seen to be feasible when Carol Lauzon and John Peloquin in a preliminary study showed that the vector insect was cycling typical plant bacteria through the midgut. The project then is to exploit this initial finding by seeking ways to deliver anti-Pierce's strategies via one of the innocuous bacteria found common to the host plant. This approach calls for expertise in environmental microbiology, plant pathology, molecular biology, entomology and microbiology. The research leaders listed above have the proper credentials to make this strategy function.

OBJECTIVES

1. Culture, identify, then genetically transform insect-associated bacteria, especially gut bacteria, from glassy-winged sharpshooter, *Homalodisca coagulata* (GWSS).
2. Find gene products that inhibit or kill *Xylella fastidiosa*, or disrupt its attachment in GWSS.
3. Work out transformation systems for the bacteria identified in No. 1 above.
4. Study the movement of plant bacteria between the vector and host plants.
5. Set up disease cycles in the greenhouse to test anti-Pierce's strategies.
6. Test the application of anti-Pierce's agents.

Realistic application of the strategies described above will require permits to release transgenic bacteria. It is obvious that studies on the effects of such organisms on the environment are called for. We are working on such permits now for use in other projects.

RESULTS AND CONCLUSIONS

The first six months of this project (initiated in March of 2001) was spent recruiting participants. Blake Bextine will join the project in mid-November. For his Ph.D., Blake worked out an artificial system to study transmission of the Yellow Vine disease of cucurbits by the squash bug, *Anasa tristis*. That disease is also caused by a bacterial pathogen identified as *Serratia marcescens*. We feel Dr. Bextine is the ideal person to work out a disease transmission protocols to test anti-

Pierce's strategies. He will work directly with Dr. Lauzon and Dr. Peloquin. Early in the project Donald Cooksey hired Ludmila Kuzina as a postdoctoral to work on rearing the pathogenic bacterium, *Xylella fastidiosa*. We were already well aware of how difficult this rearing is and are fortunate to find a young scientist willing to accept the challenge. Dr. Kuzina is testing the various anti-Pierce's gene products and agents that are identified. Dr. Lampe secured the services of a trusted technician early in the project and has been working on transformation protocols in cooperation with John Peloquin. Dr. Cooksey's other related projects are a good match to his desire to find methods of alleviating the Pierce's disease symptoms in the affected plants.

Specific research progress:

Methods of collection and isolation. Field collections of GWSS were done by trapping the insects by hand directly from vegetation in clean, fresh unused Ziploc polyethylene baggies. This was compared to the more efficient sweep net collection to determine any possible source of contamination. We went to some trouble to ensure that gut samples of bacteria were not contaminated with surface bacteria. Initial studies established that samples were indeed from the gut alone. The gut and contents were plated on nutrient agar. The inoculated agar was incubated until bacterial colonies arose. Whereupon these colonies were isolated, purified by restreaking and subsequently 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 or not 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. Besides *A. x. denitrificans*, a number of other gut bacteria were identified.

The most recent analysis of GWSS gut bacteria have included three yet to be fully characterized bacteria. *Ralstonia* was formerly known as *Alcaligenes* 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. A great deal known about the biology of *Bacillus*, and a number of dependable expression vectors are known. *Arthrobacter* species are considerably less well understood. The distribution and frequency of occurrence of these other bacteria must be further studied to understand how they may be used in a program to control Pierce's disease and/or the GWSS. Unlike *Alcaligenes xylosoxidans denitrificans*, *Chryseomonas luteola* has been very slow growing on typical laboratory media and is quite difficult to culture.

The *Alcaligenes* have thus far been found in all the GWSS collections. This suggests a possible symbiotic relationship with GWSS that also needs to be further investigated. 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. Investigation continues.

Alcaligenes xylosoxidans denitrificans strains have been characterized biochemically. The 16S rRNA sequence of JP134, *Alcaligenes xylosoxidans denitrificans* from GWSS was determined. The closest sequence to this 16S rRNA sequence matched in GenBank by BlastN was an uncharacterized *Pseudomonas* sp. In keeping with the need to study this possible aspect of the biology of these bacteria, Bruce Kirkpatrick has made arrangements with us to follow the movement (if any) of GWSS gut bacteria in plants. We hope to have a genetically marked (EGFP or similar fluorescent protein) available for him to use in his studies soon.

Transformation vectors and attempts. [pEGFP/Zeo]. This plasmid was originally used to successfully transform Gram negative endosymbionts of Tephritid gut (Peloquin, et al., 2000). An electroporation protocol developed for *Pseudomonas aerogenosa* and *Alcaligenes eutrophus* was used in attempts to transform *Alcaligenes xylosoxidans denitrificans* with this plasmid using Zeocin, a derivative of bleomycin/phleomycin as the selective antibiotic. We encountered some problems with Zeocin and are testing alternative selective antibiotics to which our isolates of *Alcaligenes xylosoxidans denitrificans* were susceptible. Kanamycin was found to be a good selective agent in disc diffusion assays. Fortunately, the GWSS bacteria grew well on the Mueller Hinton agar used for this assay.

Derivatives of pBBRMCS2. We found that our *Alcaligenes xylosoxidans denitrificans* were also susceptible to kanamycin and derivatives by growth studies on plates supplemented with this antibiotic at standard concentrations as above with Zeocin. Additionally, an analysis of *Alcaligenes xylosoxidans denitrificans* 16S rRNA sequence suggested that the closest

relatives to this bacterial ribosomal RNA in the genetic data bank were that of a *Psuedomonas* species. We then investigated alternative vectors with origins of replication and promoters different from those usually used in Enterobacteriaceae in a hope that an alternate origin of replication and antibiotic selection other than Zeocin might have better selection properties and stronger expression of transgenes. The plasmid we are developing is a derivative of the previously published pBBRMCS plasmids. We successfully transformed *Alcaligenes xylosoxidans denitrificans* isolates with plasmids derived from these elements. We hope to next engineer these plasmids so that they express useful genes for pest control. At first we will use fluorescent proteins like DsRed and EGFP. Immediately, these fluorescent protein markers can be used to trace the movements and behavior of the transformed bacteria and later to produce substances that reduce or eliminate the vector potential of GWSS infected with the transformed bacteria similarly to work done in other systems.

Endogenous mariners of the GWSS. We screened the genome of the GWSS for the presence of *mariner* transposable elements by PCR using fully degenerate primers designed against highly conserved motifs of *mariner* family transposases (Robertson 1993). PCR products were obtained with one set of the primers and the products cloned and sequenced. The GWSS contains a *mariner* that falls into the *irritans* subfamily of *mariner* transposons. All of the copies we sequenced contained multiple frame shifts indicating they were inactive. These copies are derived from a very divergent *irritans* subfamily element.

We know that the possibilities for interactions between the endogenous mariners of the GWSS and those we intend to use in the gut symbionts are likely to be non-existent for several reasons. First, there is no known way for the elements in the cells of the GWSS to interact with those inside the bacteria. Second, even if such an interaction were possible, the elements in the GWSS are inactive. Finally, the elements in the GWSS are extremely divergent in comparison to the elements proposed for use in GWSS symbionts and as such could not interact with them (Lampe et al. 2001).

A transgenic system for GWSS bacterial symbionts. We are constructing two genetic transformation systems based on the *mariner* family elements, *FamarI* and *HimarI*. The final constructs that will be used to make stable insertions into *Alcaligenes* are described below. The plasmids have several features that help it fulfill the requirements that meet our purposes. They have an RP4 origin of transfer so they can be mated from *E. coli* to *Alcaligenes*. They also have a R6K origin of replication so that they can only replicate in special strains of *E. coli*. The drug marker is carried between two FRT sites, the sequences that are used by the FLP recombinase of yeast. Thus, once insertions of the *FamarI* transposon are obtained, the drug marker can be removed by recombination. Since the transposase gene lies outside the inverted terminal repeats (ITRs), the insertions will be stable after the plasmids are lost. Using this system we should be able to obtain insertions at random positions in the chromosome of *Alcaligenes* that are completely stable. These systems are currently being tested in *Alcaligenes*.

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