# A SCREEN FOR *XYLELLA FASTIDIOSA* GENES INVOLVED IN TRANSMISSION BY INSECT VECTORS

**Project Leaders:**
Alexander Purcell
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3114
purcell@nature.berkeley.edu

Steve Lindow
Plant and Microbial Biology
University of California
Berkeley, CA 94720-3102
icelab@socrates.berkeley.edu

**Researcher:**
Clelia Baccari
ESPM-Division of Insect Biology
University of California
Berkeley, CA 94720-3114
CBaccari@nature.berkeley.edu

**Reporting Period:** The results reported here are from worked conducted September 2005 to September 2006.

## ABSTRACT
Strain KLN61 is an *rpfF* mutant strain of *Xylella fastidiosa* (*Xf*) that cannot be transmitted to plants by the sharpshooter leafhopper, *Graphocephala atropunctata* (BGSS), an efficient vector of the wild-type strain of this bacterium. This mutant was not retained and did not form biofilm in the vector mouthparts, however was hypervirulent and formed biofilm when mechanically inoculated into grapevines (Newman et al. 2004). We created additional mutants of *Xf rpfF* mutant strain, KLN61 using a transposome-mediated mutagenesis technique (Streptomycin EZ::TN custom transposome mutagenesis system). Screening of mutants for restoration of vector transmissibility revealed that strains competed during systemic colonization of grapevines, with only a single strain eventually colonizing most of the plant. This prevented our further pursuit of using vector acquisition to screen for mutants that restored vector transmissibility. We are testing whether production of a signaling molecule in the plant by the wild-type *Xf* strain would restore the transmissibility of the mutant. Two transmission experiments in which BGSS were fed sequentially on the mutant KLN61 (not vector transmissible), then on the parent wild type strain Temecula, or the reverse order, showed that either exposure sequence reduced vector transmission by 35% to over 90% compared to a single exposure to the Temecula strain alone. These unexpected results imply that feeding exposure of vectors to a mutant *Xf* strain that does not produce cell signal can reduce later transmission of normally transmissible strains, as well as reducing transmission by vectors already infectious with a transmissible strain.

## INTRODUCTION
In *Xylella fastidiosa (Xf)* the *rpf* gene system for regulation of pathogenicity factors likely regulates genes that are important for colonization and transmission by insect vectors. The *rpfF* gene is one of the essential genes of the rpf cell-cell signaling system. KLN61 which is an *rpfF* knockout, could not perform cell-cell signaling. The *RpfF* gene catalyzes the synthesis of the signaling system molecule, diffusible signal factor (DSF) (Newman et al. 2004). Importantly, while still pathogenic to grape, such strains do not colonize and hence are not vectored by sharpshooters.

The first year, our research objective was to construct a library of *Xf* transposon-disrupting mutants through transposome-mediated mutagenesis in an *Xf rpfF* mutant background. Our first approach involved creating a library in strain KLN61which could be screened for restoration of transmissibility by inoculating mutants into plants on which vectors could subsequently feed. Any mutant strains of the non-transmissible KLN61, which could be transmitted to healthy grape plants by insect vector, would have incorporated genome changes implicated in the transmission of *Xf*. The isolation and identification of these mutants would have allowed us to better understand what *Xf* genes are involved in vector transmissibility. We created a mutant library compatible with the KLN61 mutant background in order to identify other *Xf* genes involved in the complex process of transmission. We designed and constructed a Streptomycin EZ::TN custom transposome mutagenesis system in order to further mutate the *Xf rpfF* mutant strain, KLN61. While the focus of our first grant year was to create the mutant library compatible with the KLN61 mutant strain, in this last past year our focus was on screening mutants. Since the mutants are screened by placing the insect vectors in contact with the source plants that retain the mutant library we faced the question of how multiple strains coexistence would effect *Xf* movement and transmission. To our surprise this part of the research became more interesting then we anticipated (Objective 5).

## OBJECTIVES
1. Create a library of *Xf* mutants in the *rpfF* mutant background using a disrupting transposon mutagenesis to block gene function.
2. Create a library of *Xf* mutants in the *rpfF* mutant background using an activating transposon mutagenesis to enhance gene function.
3. Design and carry out a screen for disrupting transposon mutants library in *Xf* that restore transmissibility in the non-transmissible *rpfF* mutant.
4. Identify the genes affected in the screen. These will be genes that are important for the transmission of Pierce's disease (PD) by insect vectors.
5. Examine the process of colonization of plants and insect vector by co-inoculation of *Xf* Tem and *rpfF* mutant (new).

**RESULTS**

**Objective 1**

The commercially available transposome system that confers $Kan^R$ was not compatible with our KLN61 strain. In our studies we could not use this vector and had to construct a novel transposon in order for it to be compatible with our $Kan^R$ *rpfF*- mutant. Our laboratory designed and constructed a Streptomycin resistant EZ::TN transposome mutagenesis system in order to further mutate the *Xf rpfF* mutant strain. We introduced our Streptomycin resistant EZ::TN transposome in the strains KLN61 by electroporation techniques to create mutants. Electroporation with our Streptomycin resistant EZ::TN transposome yielded 5 X $10^3$ mutants per µg of DNA in *Xf* strain KLN61.

**Objective 2**

Because of efforts to create the disrupting transposon and the high yield of mutants produced with this disrupting transposon, we focused on screening the existing library, thereby postponing the construction of an activating transposon library of mutants.

**Objective 3**

To accomplish Objective 3 we designed a library of the DSF mutant transposon-generated mutants to screen using insect vector transmission. The approach was to restore transmissibility through mutagenesis by disrupting genes normally down-regulated by DSF with a disrupting transposon. Because the mutant types that we sought are those in *Xf* that restore transmissibility in the non-transmissible rpfF mutant, the screen should be effectively carried out by the insect vectors. The mutant library was needle-inoculated into the source plants. We were unable to retrieve mutants when source plants were cultured and plated on the Streptomycin selective media. This is likely due to the process of multi-strain competition during the colonization of the plants (as stated in more details in Objective 5) or to the possibility that *rpfF* Streptomycin resistant mutants were not stable in grapevines.

**Objective 4**

We couldn't carry on with Objective 4 since we were unable to retrieve mutants from source plants. However, our results in investigating strain competition as an explanation for the lack of diversity of mutants recovered gave us an interesting insight into possible explanations for strain competition among *Xf* strains in grape, which we describe below in Objective 5 (new).

**Objective 5 (new)**

In Objective 5, we are addressing the process by which the DSF signal molecule is produced and recognized by cell populations of *Xf* in plants. If the DFS signal molecule is excreted into the plant after production, it should functionally complement an *rpfF* mutant in *planta*. An understanding of how DSF is perceived by bacteria in *planta* is central to our understanding of how it affects both plant virulence factors, presumably in a density-dependent fashion, and affects insect transmission. To test this model we are interested in how RpfF+ and RpfF- strains coexist in plants and how they might affect vector transmission. Mutants of PD strains of *Xf* are relatively new and not much is known regarding their behavior with regard to transmission and coexistence in *planta*. In *vitro* experiments on solid media have shown that coexistence with wild-type can restore DSF signaling production in *Xanthomonas campestris* (Barber C. E., 1997). The purpose of these experiments was to see if the wild-type strain DSF signal is able to restore the mutant biofilm formation in the mouthparts of the vector and therefore promote transmission. To test this model, we designed transmission experiments to study insect colonization when insects were fed sequentially: first on source plants containing *Xf* Tem, and then onto plants containing the *Xf rpfF* mutant to see if the acquisition of one strain impeded or stimulated the transmission of a subsequently acquired strain.

A total of 90 BGSS free of *Xf,* were divided into two groups of 15 insects each for use as negative controls and two groups of 30 insects each for the experimental treatments. The groups which held 30 BGSS were placed in contact with grapevines infected with Tem containing high population of bacteria for three days to allow the BGSS to acquire *Xf*. We then transferred the insects for another three days feeding period on a symptomatic vine infected by KLN61 and containing high population of the mutant. At the same time we also transferred the insects fed first on KLN61 vines for another three days feeding period on a symptomatic vine infected by Tem. After the BGSS had fed sequentially for three days on each source plant, they were transferred to healthy seedlings, which were tested for confirmation of transmission after eight weeks in the greenhouse. After seven days on healthy grape test plants, the BGSS were removed from the plants, the head of the insects were severed, cultured and plated on PWG media and PWG+Kan media. (Figure 1).

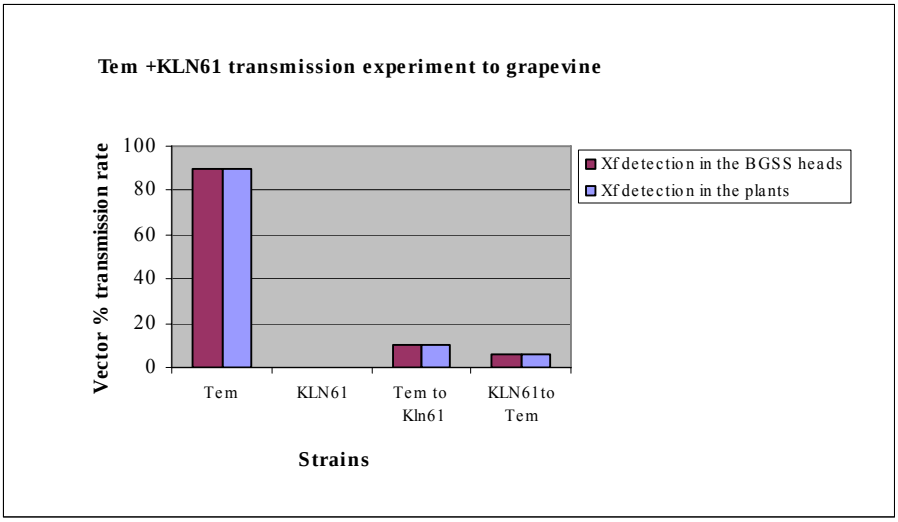


**Figure 1.** *Xf* Tem and KLN61 transmission to grapevines.

In the first experiment the BGSS groups had been exposed to different source plants, even though all the source plants were infected with the same *Xf* strain. In order to avoid the possibility that transmission results were due to differences among individual source plants in their efficiency as sources for vector acquisition, in the second experiment, we exposed the insects at the same time on the same plants. This ensured that the results were due only to the bacterial strains used and not to variations among the source plants. A total of 60 greenhouse reared *Xf*-free BGSS individuals were divided into four groups of 15 insects. The insects were pre-screened as in the first experiment, divided into groups of five, and caged on different parts of the symptomatic source plants. There were a total of four source plants, two infected with KLN61 and two infected with the Temecula strain. After a three day acquisition feeding period, a group of five BGSS (a total of 20) were removed from each of the source plants, representing the positive controls. The remaining insects were switched to the opposite strain source plants for a three-day period (total of six days of acquisition time). The BGSS were then individually transferred to healthy seedlings for a seven-day IAP. After eight weeks in the greenhouse, the seedlings were tested by culturing to confirm transmission. After seven days on healthy test vines, the insects were removed from the plants and prepared as in the first experiment. The heads were cultured and plated on PWG media and on PWG+Kan media (Figure 2).

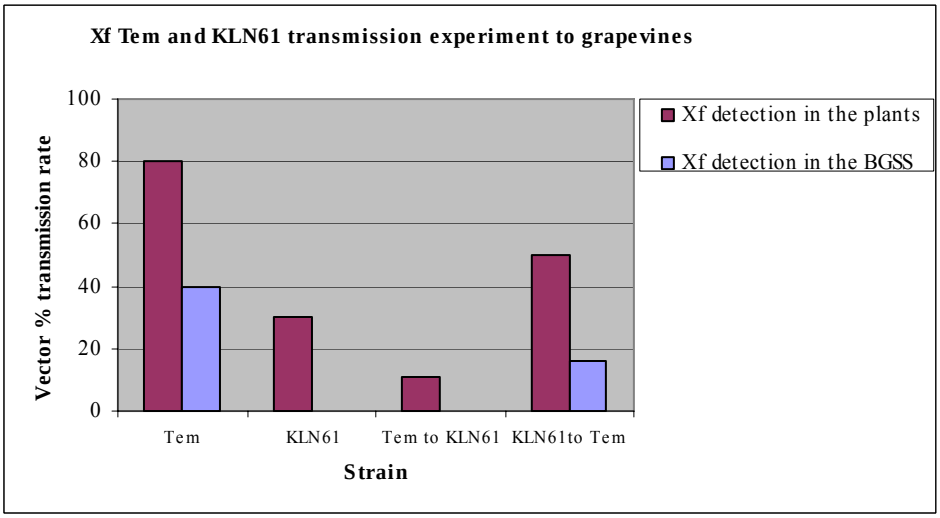


**Figure 2.** *Xf* Tem and KLN61 transmission to grapevines.

It was surprising that none of the strain combination exposures restored the mutant bacteria transmission. More importantly, when BGSS were exposed to source plants containing the wild type strain first and then source plants containing KLN61, they were unable to transmit the wild type. More experiments are undergoing in our laboratory to further study these interesting phenomena.

## CONCLUSIONS

The results of vector transmission experiments to test strain competition between the wild type *Xf* Tem and the non-transmissible *rpfF* mutant were very interesting. We found that one strain inhibited the vector acquisition/transmission of a second strain. These results should have implications for understanding the epidemiological consequences of strain

competition in *Xf*. Because these results are still preliminary and contrary to our expectations, we are repeating them and expanding experiments based on the continuing results.

The implications of our findings relative to strain competition could be useful in better understanding how *Xf* colonizes grapevines. This has broad implications for many other physiological and anatomical studies of PD. Strain competition could also in part explain the effects of biological control (or cross-protection) of PD with *Xf* strains that are not pathogenic in grape (Hopkins 2005). We have begun greenhouse experiments that may elucidate this phenomenon using *Xf* strains from California (Almeida and Purcell 2004) that multiply in grape without causing PD symptoms to see if their prior colonization of plants can prevent infection by typically virulent PD strains.

**REFERENCES**

Hopkins, D. L. 2005. Biological control of Pierce's disease in the vineyard with strains of *Xylella fastidiosa* benign to grapevine Plant Disease 89 (12):1348-1352.

Newman, K. L., R. P. P. Almeida, A. H. Purcell and S. E. Lindow. 2004. Cell-cell signaling controls *Xylella fastidiosa* interaction with both insects and plants Proc. Nat. Acad. Sci. 101: 1737-1742.

Newman, K. L., R. P. P. Almeida, A. H. Purcell and S. E. Lindow. 2003. Use of a green fluorescent strain for analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*. Appl. Environ. Microbiol. 69:7319-7327.

Dow, J.M. and M.J. Daniels. 2000. *Xylella* genomics and bacterial pathogenicity to plants. Yeast 17: 263-271.

Simpson, A.J.G, F.C. Reinach, P. Arruda, 97 other authors. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406: 151-157.

Barber,C. E., J. L. Tang, M. Q. Feng, M. Q. Pan, T. J. G. Wilson, H. Slater, J. M. Dow, P. Williams and M. J. Daniels. 1997. A novel regulatory system required for pathogenicity of *Xanthomonas campestris* is mediated by small diffusible signal molecule. M. M.24 (3): 555-566.

**FUNDING AGENCIES**

Funding for this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board, and by the California Agricultural Experiment Station of the College of Natural Resources, University of California, Berkeley.

# DOCUMENTATION AND CHARACTERIZATION OF *XYLELLA FASTIDIOSA* STRAINS IN LANDSCAPE HOSTS

**Project Leaders:**

Frank Wong
Dept. of Plant Pathology
University of California
Riverside, CA 92521
frank.wong@ucr.edu

Donald A. Cooksey
Dept. of Plant Pathology
University of California
Riverside, CA 92521
donald.cooksey@ucr.edu

Heather S. Costa
Dept. of Entomology
University of California
Riverside, CA 92521
heather.costa@ucr.edu

Rufina Hernandez
Dept. of Plant Pathology
University of California
Riverside, CA 92521
rufinah@ucr.edu

**Reporting Period:** The results reported here are from work conducted October 1, 2005 to September 30, 2006.

## ABSTRACT

To document the incidence of *Xylella. fastidiosa* (*Xf*) in landscape ornamental hosts, in 2003 and 2004 a survey of plants showing symptoms of scorch or dieback in urban locations in southern California was done. A total of 1,670 samples, representing 161 plant species were taken and analyzed at five locations: Fillmore, San Diego, Redlands, Riverside, and Tustin. From the total, 35% of plants tested (591), representing 102 identified species, gave positive results by *Xf*-specific enzyme linked immunosorbent assay (ELISA). Isolation of bacteria from ELISA-positive plants provided 39 isolates form 14 non-previously reported as *Xf*-hosts species: almond, crapemyrtle, daylily, gingko, jacaranda, grapevine (both *labrusca* and *vinifera*), magnolia, mulberry, oleander, cherry, purple-leaved plum, heavenly bamboo, olive, sweetgum, plum and western redbud. Random amplified polymorphic (RAPD)- polymerase chain reaction (PCR) and sequence analysis of the 16S-23S rDNA intergenic spacer regions (ISR) was used to genetically characterize the strains. Strains isolated from daylily, jacaranda and magnolia grouped with members of *Xf* subsp. *sandyi*. Some strains isolated from cherry, and one strain isolated from western redbud, grouped with *Xf* subsp. *fastidiosa* members and strains isolated from purple-leaved plum, olive, peach, plum, sweet gum, maidenhair tree, crape myrtle and another western redbud strain, clustered with members of the *Xf* subsp *multiplex*. All strains isolated from mulberry and one from heavenly bamboo formed a cluster that has not yet been defined as a subspecies. Koch's postulates were successfully tested for the strains isolated from sweet gum, purple-leaved plum, western redbud and mulberry. Cross-infectivity of those strains to grapevine, almond and oleander was also tested. This information contributed to better understand the role of these different strains in causing disease on plants in urban landscapes. However, the impact of *Xf* infections in landscape hosts and the diversity of strains still are far from being fully understood.

## INTRODUCTION

*Xylella fastidiosa (Xf)* is a xylem-limited, insect-vectored, plant pathogen that can cause severe damage to a wide range of host plants including grape, almond and oleander. In addition to causing Pierce's disease (PD), Almond leaf scorch (ALS) and Oleander leaf scorch (OLS), *Xf* has been implicated in causing bacterial leaf scorch in a number of ornamentals and trees in the mid-Atlantic and southeastern U.S. Affected plants include oak, sycamore, elm, mulberry, maple and other shade trees in the landscape and urban forests (Sherald and Kostka 1992, McGovern and Hopkins 1994, McElrone et al. 1999). Multiple strains of *Xf* with different host ranges have been identified (Chen et al. 1992, da Costa et al. 2000, Hendson et al. 2001), but little is known about the diversity of these populations in the urban landscape and their ability to cause loss in plants of horticultural and agronomic importance. The arrival of a highly efficient vector, the glassy-winged sharpshooter (*Homalodisca vitripennis*) in California has resulted in the rapid spread of this pathogen amongst both agricultural crops and landscape plants. Both PD and OLS are present in this area and recently, disease symptoms have been associated with the presence of partially characterized and potentially new strains of *Xf* in a number of landscape ornamentals including olive, liquidambar and purple-leaved plum. The broad host range of *Xylella* and its ability to hide inside unaffected hosts make it a constant menace for agricultural crops. Very little was known previously about the fate of *Xylella* in ornamentals, the strains they are harboring and their ability to cause disease losses in plants of agronomic importance. To find some information in this subject, we isolate and characterized strains from ornamental hosts. Our studies identified new hosts for the *Xf* subspecies *fastidiosa*, *Xf* subspecies *multiplex*, *Xf* subspecies *sandyi*, and for the mulberry leaf scorch type strains. Some strains appear to have a very limited host range and some have a broader range of hosts, but for most strains the possible host-strain combination has not been extensively tested.

## OBJECTIVES

1. Identification of landscape host species infected with *Xf*.
2. Genetic characterization of the strains of *Xf* isolated from landscape plant species.
3. Confirmation of pathogenic infection through inoculation studies with specific isolates.
4. Test ability of new strains to infect established host plants of *Xf* including grape, oleander and almond.