ANALYSIS OF *XYLELLA FASTIDIOSA* TRANSPOSON MUTANTS AND DEVELOPMENT OF PLASMID TRANSFORMATION VECTORS

Project Leader:
Bruce Kirkpatrick
Dept. of Plant Pathology
University of California
Davis, CA 95616

Cooperator:
Magalie Guilhabert
Dept. of Plant Pathology
University of California
Davis, CA 95616

Reporting Period:

ABSTRACT
We screened over 1,000 random Tn5 *Xylella fastidiosa (Xf)* mutants in Chardonnay grapevines growing in the greenhouse in 2003. Approximately 10 of the mutants exhibited a hypervirulent phenotype, i.e. vines inoculated with these mutants developed symptoms sooner and died sooner than vines inoculated with the wild type *Xf* parental strain. The identity of the Tn5 insertion sites in these mutants was reported at 2003 PD Symposium. In 2004 we re-inoculated these hypervirulent mutants into another set of Chardonnay, Chenin blanc and Thompson seedless vines and the hypervirulent phenotype was reproduced in all 3 varieties. Movement and populations assays showed that the hypervirulent mutants moved faster and reached higher populations than wild type *Xf*. In the first Chardonnay screen, we identified an unexpectedly high number of avirulent mutants. Because some of these may have been the result of poor inoculation we sequenced the DNA that flanked the Tn5 insertion in all the mutants. Those mutants with Tn5 insertions in genes other than "house keeping" genes were re-inoculated into a new set of vines and their pathogenic phenotype is being determined. Additional small (1.3kb) native *Xf* plasmids were engineered as potential *Xf/E. coli* shuttle vectors. However, like our other similar constructs, these plasmids were not stably maintained without antibiotic selection, and not useful tools for *in planta* gene complementation studies.

INTRODUCTION
During the past 4 years one of the objectives of our research on Pierce's disease (PD) has involved the development of transformation and transposon mutagenesis systems for the bacterium that causes Pierce's disease (PD), *Xylella fastidiosa* (*Xf*). We developed a random transposon based mutagenesis system for *Xf* in 2001 (Guilhabert et. al., 2001). Recently, we developed two *E.coli/Xf* plasmid shuttle vectors, one based on the plasmid RSF1010 and the other based on a small cryptic plasmid found in one of the grapevine *Xf* strains, UCLA. Both those plasmid shuttle vectors replicate autonomously in *Xf* (Guilhabert and Kirkpatrick, 2003; Guilhabert and Kirkpatrick, manuscript submitted for publication). However these plasmids are only stably maintained in *Xf* cells that are kept under selection using the antibiotic, kanamycin. Therefore, these vectors will be useful for *in vitro* studies of *Xf* gene function; however they cannot be used to study the function of *Xf* genes in the plant host. We evaluated other plasmids that can be stably maintained in *Xf* cells inoculated into plant hosts.

The complete genome sequence of a citrus (Simpson et al., 2000) and a grape (Van Sluys et al., 2002) strain of *Xf* have been determined. Analysis of their genomes revealed important information on potential plant pathogenicity and insect transmission genes. However, approximately one-half of the putative ORFs that were identified in *Xf* encode proteins with no assignable function. In addition, some of the putative gene functions assigned on the basis of sequence homology with other prokaryotes may be incorrect. For these reasons we felt that it was important to develop and assess the pathogenicity of a library of random Tn5 mutants in order to identify <u>any</u> gene that may influence or mediate *Xf* pathogenicity. Our group, as well as other PD researchers, is evaluating specific mutants in *Xf* genes that are speculated, based on homology with other gene sequences in the database, to be involved with pathogenicity. However, screening a random transposon (Tn) library of *Xf,* a strategy that has led to the identification of important pathogenicity genes in other plant pathogenic bacteria, may identify other novel genes, especially those that regulate the expression of pathogenicity/attachment genes that will be important in the disease process. Using Tn5 mutagenesis, there is a high probability that we can knock out and subsequently identify *Xf* genes that mediate plant pathogenesis. Proof that a particular gene is indeed mediating pathogenicity and/or insect transmission would be established by re-introducing a cloned wild type gene back into the *Xf* genome by homologous recombination, or more ideally, introduce the wild type gene back into *Xf* on the plant stable shuttle vector.

OBJECTIVES
1. Screen a library of *Xf* transposon mutants for *Xf* mutants with altered pathogenicity, movement or attachment properties.
2. Identify and characterize anti-virulence *Xf* genes.
3. Identify and characterize virulence *Xf* genes.
4. Develop a *Xf/E. coli* transformation plasmid that is stable *in planta*

RESULTS AND CONCLUSION
Objective 1
Using the transposome technology previously described (Guilhabert et al., 2001) we obtained 2000+ *Xf* Tn5 mutants, which should represent fairly random mutagenesis events throughout the *Xf* genome. During the spring and summer 2002, we inoculated 1,000 chardonnay plants with individual *Xf* Tn5 mutants using a pinprick inoculation procedure (Hill and Purcell,

1995; Purcell and Saunders, 1999). The vines were grown in pots in a greenhouse using a nutrient-supplemented de-ionized drip irrigation system. The parental, Temecula strain served as a positive control and a water inoculation served as a negative control. Two months after inoculation, the vines were observed for symptom development approximately every two weeks for 6 more months (32 weeks total after inoculation). The symptoms were rated on a visual scale from 0 to 5, 0 being healthy and five being dead. Rating of 1 showed only one or two leaves with the scorching symptom starting on the margins of the leaves. Rating of 2, showed two to three leaves with more developed scorching. Rating of 3 showed all the leaves with some scorching and a few attached petioles whose leaf blades had abscised (match sticks). Rating of 4 showed all the leaves with heavy scorching and/or numerous match sticks.

We successfully identified *Xf* mutants with altered virulence, confirming for the first time, that screening a library of Tn5 *Xf* mutants in susceptible hosts can identify genes mediating *Xf* pathogenicity. We also developed a two-step procedure, direct PCR on *Xf* colony and direct sequencing of the PCR product that can rapidly identify *Xf* Tn5 insertion sites.

Objective 2

Six months after inoculation (see objective 1), 10 of the inoculated Chardonnay vines showed hyper-virulence, i.e. more severe symptoms compared to the vines inoculated with wild type *Xf* cells. This phenotype was further confirmed in Chenin Blanc and Thompson Seedless grapevines. Further analysis demonstrated that all the hypervirulent *Xf* mutants tested showed i) earlier symptom development, ii) higher disease scores over a period of 32 weeks and iii) earlier death of inoculated grapevines than vines inoculated with wild type; thus demonstrating that the hypervirulence phenotype is correlated with earlier symptom development and earlier vine death in multiple *Vitis vinifera* cultivars. The hypervirulent mutants also moved faster than wild type in grapevines. These results suggest that i) wild type *Xf* attenuates its virulence *in planta* and ii) movement is important in *Xf* virulence. The mutated genes were sequenced and their insertion sites confirmed by PCR amplification and sequencing of PCR products. None of the mutated genes had been previously described as anti-virulence genes, although six of them showed similarity with genes of known functions in other organisms. The hypervirulent mutants were further characterized for *in vitro* and *in planta* attachment. One of the hypervirulent mutants was altered in its microcolony formation and biofilm maturation within the xylem vessels (Figure 1). We are in the process of further characterizing the protein involved in *Xf* biofilm maturation.

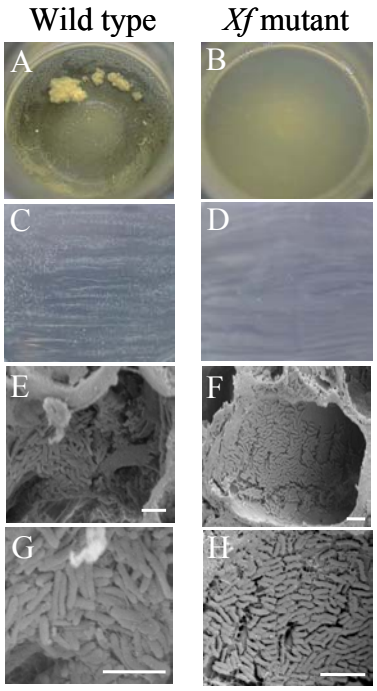

Figure 1: A hypervirulent *Xf* mutant showns a lack of microcolony formation and biofilm formation. Panels A-G are *Xf* wild type cells; Panels B-H are *Xf* mutant cells. Panels A and B wild type and mutant cells, respectively, inoculated into PD3 medium in a 125 mL flask and placed on a shaker. The degree of self-aggregation was visualized after 10 days of incubation. Panels C and D wild type and mutant cells, respectively, plated onto PD3 medium plates. The colony morphology was examined after 10 days of incubation. Panels E and F, wild type and mutant cells in xylem vessels. Note the lack of a three dimension array in the mutant compare to wild type. Panels G and H, close up of wild type and mutant cells in a biofilm. Note the wild type cells typically aggregated together side to side while the mutant cells did not aggregate in this manner. Scale bar equivalent to 5 microns in every panel.

Table 1: Function categories of *Xf* DNA flanking Tn5 transposon insertion in putatively avirulent *Xf* mutants

Putative Gene function	% of Mutants Affected
Hypothetical protein	29
House-keeping	26
Phage-related protein	20
Pathogenicity/virulence	10
Intergenic region	6
Surface protein	2
Transporter	2
Regulator of transcription	1
Mobility	1
Transposon elements	1
Cell-Structure	1
Undefined category	1

Objective 3

Six months after inoculation (see objective 1), we also noticed an unexpectedly high percentage (35%) of inoculated vines that did not develop typical PD symptoms. One might have expected no more than 5% or so of the mutants to be non pathogenic. We sequenced the *Xf* DNA, flanking the Tn5 element in order to determine the specific location of the Tn5 insertion in each putatively "avirulent" mutant. Table 1 summarizes the categories of the genes that were knocked out in the avirulent *Xf* mutants. We then chose to further characterize insertions in open reading frames (ORFs) that code for proteins that have possible roles in *Xf* virulence/colonization or ORFs with no known function. Tn5 insertions in known "house-keeping" genes were not screened further. Three new Chardonnay grapevines growing in pots in the greenhouse were inoculated with each *Xf* mutant of interest as well as the appropriate controls. The experiment was done in duplicate. The rate of symptom development or lack there of, is being monitored as we described in objective 1. After 14 weeks, petiole samples at the point of inoculation (poi) and 12 inches above the poi will be taken from each mutant and control vines. *Xf* cells will be cultured from those samples in order to assess bacterial population and colonization. The insertion sites will be further confirmed by PCR.

Objective 4: Develop a Xf/E. coli Shuttle that is Stable in planta.

A plasmid DNA fraction was isolated from the UCLA strain of *Xf* and subjected to *in vitro* mutagenesis using the transposome technology that was previously used to create our Tn5 *Xf* library. This DNA was electroporated in the UCLA strain and 4 kan^R colonies were obtained. These were sequenced and found to be insertions in the small 1.3kb plasmid that we previously attempted to develop as a *Xf/E. coli* shuttle vector. These Tn5 insertions were in different areas of the native plasmid so we tested the relative stability of these plasmids by culturing the transformants on PD3 medium with and without kanamycin. After 3 passages on non-selective media the colonies were transferred to PD3 media containing kanamycin and no colonies were observed on the plates. This indicates that the plasmids containing the Tn5 insertions were lost upon culture in non-selective medium, results that were the same as our previous attempts to engineer these small native plasmids as shuttle vectors. Future work will focus on a similar strategy to construct a shuttle vector from the 5.8kb plasmid in the UCLA strain, with the hope that this construct might be stably maintained in *Xf* without antibiotic selection.

REFERENCES

Guilhabert, M. R and Kirkpatrick, B. C. 2003. Transformation of *Xylella fastidiosa* with broad host range RSF1010 derivative plasmids. Mol. Plant Path. 4, 279-285.

Guilhabert, M. R., Hoffman, L. M., Mills, D. A. and Kirkpatrick, B. C. 2001.Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. Mol. Plant-Microbe Interact. 14:701-706.

Hill, B. L. and Purcell, A. H. 1995. Multiplication and movement of *Xylella fastidiosa* within grapevine and four other plants. Phytopath. 85, 1368-1372.

Purcell, A. H. and Saunders, S. R. 1999. Fate of Pierce's disease strains of *Xylella fastidiosa* in common riparian plants in California. Plant Dis. 83, 825-830.

Simpson, A. J. G. et al. 2000. The genome sequence of the plant pathogen *Xylella fastidiosa*. Nature 406: 151-157.

Van Sluys et al., 2003. Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated cholorosis strains of *Xylella fastidiosa*. J. Bacterial. 185, 1018-1026.

FUNDING AGENCIES

Funding of this project was provided by the CDFA Pierce's Disease and Glassy-winged Sharpshooter Board and the University of California Pierce's Disease Grant program.