ANALYSIS OF *XYLELLA FASTIDIOSA* TRANSPOSON MUTANTS AND DEVELOPMENT OF AN *EN PLANTA XYLELLA FASTIDIOSA* PLASMID VECTOR

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ABSTRACT
We have developed an autonomously replicating *Xylella fastidiosa* (*Xf)/E. coli* plasmid that efficiently transforms *Xf*; unfortunately this plasmid was not stably maintained in *Xf* cells *en planta* or without antibiotic selection *in vitro*. Another plasmid, containing an *Xf* native plasmid, a Kan^R cassette and cloned in pUC18 was also constructed and shown to be unstable without antibiotic selection. 1000 random Tn5 mutants were again screened for virulence in grapevines growing in the greenhouse. An expectedly high percentage (35%) did not develop typical pierce's disease (PD) symptoms; the insertion sites of the Tn5 in these apparently avirulent mutants are being sequenced. Approximately 3% of the random mutants were hypervirulent as compared to the wild type parental strain. Insertion sites of Tn5 in these mutants showed 2 were in putative LPS genes and 1 was in a hemagglutinin-like gene. The phenotype of these putative hypervirulent mutants is being confirmed in a second round of grapevine inoculations.

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 PD, *Xylella fastidiosa* (*Xf*). We developed a random transposon based mutagenesis system for *Xf* in 2001 (Guilhabert et. al., 2001). Recently, we developed an *E.coli/Xf* plasmid shuttle vector based on the plasmid RSF1010 that replicates autonomously in *Xf* (Guilhabert and Kirkpatrick, 2003), however this plasmid is only stably maintained in *Xf* cells that are kept under selection using the antibiotic, kanamycin. Therefore, this shuttle vector will be useful for *in vitro* studies of *Xf* gene function; however it cannot be used to study the function of *Xf* genes in the plant host. We continue to evaluate 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 also 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 we propose to develop.

OBJECTIVES
1. Develop a *Xylella fastidiosa* (*Xf*)/*E. coli* plasmid shuttle vector that is stable *en planta.*
2. Screen a library of *Xf* transposon mutants for *Xf* mutants with altered pathogenicity, movement or attachment properties**.**

RESULTS AND CONCLUSION
<u>Objective 1</u>
We are cloning the RSF1010 origin of replication into pUC18, creating a plasmid harboring a polylinker, a LacZ selection and expressing the pUC18 antibiotic cassette, carbenicillin that was showed to be expressed in an *Xf* background (Qin and Hartung, 2001). Such vector will allow selection for plasmid maintenance using an antibiotic that is different from the kanamycin resistance gene carried on the transposome that we use to create Tn5 *Xf* mutants and provide the necessary tool to complement and prove the function of a Tn-tagged *Xf* gene.

Another approach to create an *Xf* plasmid shuttle vector is to modify native *Xf* plasmids with a selectable marker. Such a strategy was successful in developing a stable plasmid vector for citrus (CVC) infecting strains of *Xf* (Qin and Hartung, 2001) however this plasmid (pER10) did not replicate in grape strains of *Xf* (Guilhabert and Kirkpatrick, 2003). A 1.3 kb size plasmid from a PD *Xf* strain was cloned in pUC18 and sequenced. Nucleotide and amino acid sequence analysis revealed conserved sequences that are typical of initiator (Rep) proteins involved in rolling-circle type DNA replication as well as a

putative origin of replication (Guilhabert and Kirkpatrick 2000). The kanamycin resistance cassette carried by the transposome was cloned into the multiple cloning site of the pUC18, creating plasmid pXF001. Plasmid DNA was electroporated in *Xf* in the presence of a TypeOne™ Inhibitor (Epicentre Technologies, Madison, WI) and *Xf* transformants were obtained using the Fetzer, Traver and Temecula strains. Plasmid DNAs were extracted from Kan^R *Xf* colonies and no plasmid DNAs were visualized on a gel after electrophoresis on a gel. The plasmid DNA extracted from Kan^R *Xf* cells was used to transform *E. coli* cells. Plasmid DNAs of same size as the original pXF001 were visualized on a gel (Figure1). These results indicate the ability of the pXF001 to replicate autonomously in *Xf*. Maintenance of pXF001 in *Xf* was measured in absence of antibiotic selection. As showed in Table 1, pXF001 is not stably maintained in Fetzer, Temecula or Traver strains without antibiotic selection. However, stability in *Xf* strains harboring other plasmids (Traver) seems higher than in the strain harboring no plasmid (Fetzer). We are currently evaluating the UCLA strain harboring 4 plasmids (Hendson et al., 2001) for transformation with pXF001 and maintenance without antibiotic selection.

Objective 2

Using the transposome technology previously described, 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 growing in pots in this greenhouse with the *Xf* Tn5 mutants. Four month after inoculation, we 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 will sequence the *Xf* DNA that flanks the Tn5 element in order to determine the specific location of the Tn5 insertion in each mutant that we plan to further characterize. Insertions in open reading frames (ORFs) that code for proteins that have possible roles in plant colonization or ORFs with no known function will be further characterized, while Tn5 insertions in known "house-keeping" genes will not be immediately screened further.

Three percent of the inoculated vines showed hypervirulence when compared to the vines inoculated with wild type *Xf* cells. The insertion sites of 9 of 13 mutants were sequenced and the result is summarized in Table 2. The hypervirulent mutants will be further characterized for bacterial populations in grapevines and *in vitro* attachment to glass, cellulose and chitin substrates.

Table 1. Maintenance of pXF001 plasmid in *Xf* strains under non-selective conditions of growth[a]

Xf strains	*Xylella fastidiosa*	
Number of passages	1	3
Fetzer	1	0
Traver	66	ND[b]

[a]Expressed as the percentage of Kan resistant clones after 1 or 3 generations
[b]Not done

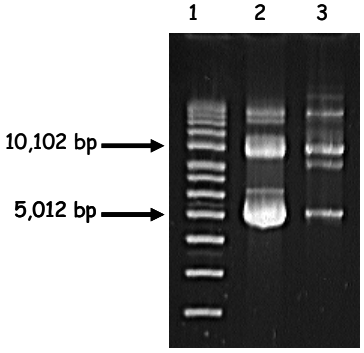

Figure 1. Gel electrophoresis of pXf001 DNA isolated from *E. coli.* and *Xf.*

Table 2. Sequence analysis & putative function of *Xf* DNA flanking Tn5 transposon insertions in hypervirulent *Xf* mutants.

Tn5 Clone	ORF designation[a]	Map location of *Xf* PD strain[b]	Putative gene function[c]
02.18. B3	1771.2	267360-267914	O-antigen lipopolysaccharides synthesis
02.18. F7	13981.2	1788832-1787882	O-antigen lipopolysaccharides synthesis
04.03. C4	19301.2	2512329-2501959	hemagglutinin-like secreted protein
03.06A C8	6371.2	852374-854482	dipeptidyl aminopeptidase
02.30. G4	11011.2	1394890-1397307	ferric enterobacyin receptor
02.30 C9	22291.2	1087319-1085869	hypothetical protein
03.06A B10	23541.2	1446631-1446398	hypothetical protein
03.06A B4	-[d]	-[d]	hypothetical protein in CVC
02.08 E10	-[d]	-[d]	hypothetical protein in CVC
02.15 B1	ND	ND	ND
03.06A E3	ND	ND	ND
03.06A G2	ND	ND	ND
02.30 B7	ND	ND	ND

[a] Identification number of open reading frame (ORF) in PD strain of *Xf*
[b] Numbers indicate the position of the Tn5 transposon in the genomic sequence of PD strain of *X*
[c] Putative function of ORF based on homology with other gene sequences
[d] No corresponding ORF in the PD strain. However, ORFs were found in the CVC strain

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