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

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ABSTRACT
A strategy is being pursued that will identify genes involved in insect transmission of *Xylella fastidiosa* (*Xf*) by both gain of function and loss of function mutagenesis of a *Xf* mutant that is unable to be transmitted by sharpshooters. KLN61 is an *rpfF* mutant strain of *Xf* (Newman et al. 2004) that cannot be transmitted to plants by the sharpshooter leafhopper, *Graphocephala atropunctata*, a common vector of the wild-type strain of this bacterium. This mutant, which did not form biofilm and was not retained in the vector mouthparts (Figure 1), was hypervirulent and formed biofilm when mechanically inoculated into grapevines (Newman et al. 2004). In this work, we are further investigating the *rpfF* mutant non-transmissible phenotype.

A molecular approach is being used to further mutate this mutant using a transposome-mediated mutagenesis technique. 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 successfully constructed a Streptomycin EZ::TN custom transposome mutagenesis system in order to further mutate the *Xf rpfF* mutant strain, KLN61. We introduced our transposome into KLN61 by electroporation, yielding 5 X 10^3 mutants per μg of DNA. To date we have mechanically inoculated grapevines with about a thousand mutants.

In complementary studies we are examining the process of colonization of plants by *Xf* and determining the extent to which cell-cell communication via signal molecule production occurs *in planta* in *Xf*. 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. The finding that co-inoculations with two different *Xf* strains results in infections in which each stain is equally likely to be found as the dominant strain suggests that Pierce's disease (PD) is characterized by a process by which many sequential occurrences of movement of a few cells to neighboring xylem vessels occurs in the process of colonizing grape plants.

INTRODUCTION
In *Xf,* the rpf (regulation of pathogenicity factors) system 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 r*pfF* gene catalyzes the synthesis of the signaling system molecular DSF (diffusible signal factor) (Newman et al. 2004). Importantly, while still pathogenic to grape, such strains do not colonize and hence are not vectored by sharpshooters (Figure 1).

In order to understand the function of other *Xf* genes involved in the complicated process of transmission, it is important to use techniques that knock out and consequently make possible the identification of related genes. Transposome-mediated mutagenesis is an effective tool for this purpose.

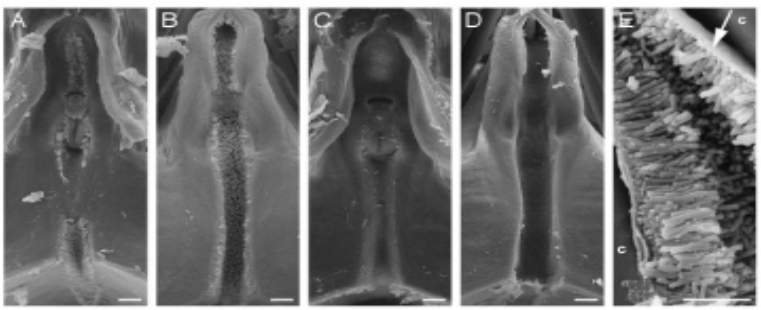

Figure 1. A, B , E: leafhopper foreguts colonized with wild type *Xf* acquired from infected plants. C, D: lack of biofilm in leafhoppers that probed plants with *rpfF* mutant (from Newman et al. 2004)

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 allow us to better understand what *Xf* genes are involved in vector transmissibility.

In complementary studies, we are addressing the process by which DSF signal molecule is produced and recognized by cell populations in plants. If DSF signal molecule is actually excreted into the plant after production it should enable functional complementation of 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 this experiment is 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.

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 mutations 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 transmission of PD by insect vectors

RESULTS

Objective 1

It has been shown that transposome-mediated mutagenesis was successful in the Kirkpatrick's laboratory when applied to wild type *Xf* (Guilhabert et al. 2001). The commercially available transposome system confers Kan^R, which 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 successfully constructed a Streptomycin resistant EZ::TN transposome mutagenesis system in order to further mutate the *Xf rpfF* mutant strain. We introduced our Strep EZ::TN Transposome in the strains KLN61 by electroporation techniques to create mutants. Electroporation with our Strep EZ::TN Transposome yielded 5 X 10^3 mutants per µg of DNA in *Xf* strain KLN61.

Objective 2

Because of the high yield of mutants produced with this disrupting transposon we have opted to focus our work on screening the existing library, thereby postponing the construction of an activating transposon library of mutants.

Objective 3

The first step of the screening of mutants has begun. Mutants have been needle-inoculated into the plants that will be used as source plants for transmission experiments. The source plants are starting to show symptoms. We also have begun culturing the source plants for monitoring bacteria population. The mutants are screened by placing the insect vectors in contact with the source plants that retain the mutant library. The *Xf*-free insect vectors (blue-green sharpshooter (BGSS) and glassy-winged sharpshooter (GWSS)) will be fed on the grapevines containing the mutant collection. The *Xf* that has regained the ability to be retained and transmitted by the insect vector, to a healthy plant seedling, will be then identified.

With the intention of testing if the wild-type strain could restore transmissibility in the *rpfF* mutant strain we have co-inoculated a total of 47 cuttings of Cabernet Sauvignon with an equal mixture of both strains or with only a single strain, in two separate experiments. We needle co-inoculated grape cuttings with a suspension of Temecula (wild-type), KLN61 (*rpfF-/Kan* resistant) *Xf,* or a mixture of equal proportions of each (C. Baccari and C. Wistrom) (Table 1).

Table 1. Inoculations of Cuttings

Set I		Set II	
Treatment	No. vines inoculated	Treatment	No. vines inoculated
Temecula	10	Temecula	6
KLN61	10	KLN 61	6
Tem+KLN61	9	Tem + 61	6

Confirmation of infection will be tested by culturing symptomatic petioles from the plants. Growth of *rpfF* and Tem will be tested by dilution plating on culture media and PCR. *Xf RpfF* is Kan^R and can grow on kanamycin selective media whereas

wild-type Temecula grows only on un-amended PWG. Colonies that grow on plain PWG can be replica plated onto PWG media with 30µg/l kanamycin, and the number of transferred colonies will be compared. Populations will be estimated via dilution plating and counted with a stereoscope. When source plants have reached a high bacteria population they will be used for transmission experiments. BGSS and GWSS will be fed on the source plant containing both *rpfF* and Tem. By culturing and PCR we will be able to distinguish both or one of the strains in the insect vector mouthparts. The insect vectors will then be placed on healthy seedlings for transmission.

Of 15 Cabernet Sauvignon plants we infected with a mixture of both strains, 46% became infected with only KLN61 and 20% with only wild type Temecula. The remaining 34% of the plants were not infected with either strain. On the first set only KLN61 was recovered from plants co-inoculated with both Temecula and KLN61 after four weeks. Populations of KLN61 in plants co-inoculated with Temecula as well as in plants inoculated only with KLN61 were approximately equal after two (8.5×10^4 and 1.0×10^5, respectively) and four (1.5×10^6 and 8.5×10^6) weeks. In the second set, 50% of the co-inoculate plants contained KlLN61 only and 50% contained Temecula only, after eight weeks. More culturing is in progress, on both set of plants, to further survey the bacteria populations.

Our preliminary results are strongly supportive of a model of progressive and sequential colonization of a large number of xylem vessels after inoculation of a single vessel by *Xf*. It is generally agreed that symptoms of PD do not occur until large numbers of vessels are colonized by *Xf*. Studies by Newman et al. 2003 found a very high correlation between incidence of highly colonized vessels and symptom development in grape. Thus *Xf* must move through many (perhaps hundreds) of different xylem cells before such high levels of colonization were to occur. If only a few cells were transferred to adjacent xylem vessels as suggested by the microscopy analyses of Newman et al. 2003, then with time it is likely that only one genotype of an originally mixed genotype inoculum might be present after such large numbers of "bottlenecks" incurred during movement in the plant. These studies are the first to support such a model and thus provide new insight into the infection process.

Objective 4

When restoration of transmission by the insect vectors occurs, we will determine what genes were affected in the screen that resulted in restored transmission. To establish which genes, when inactivated, restored the ability of the *rpfF*- strain to be transmitted by sharpshooters were affected, we will clone and sequence the transposon DNA flanking, using standard molecular biology protocols. This will determine were the transposition insertion occurred in the *Xf* genomic DNA and which particular genes were involved in the process.

CONCLUSIONS

Past experiments with the *rpfF*- mutant KLN61 (Newman et al.2004) have shown that this mutant is hypervirulent and capable of forming biofilm in grapevines. More studies on this subject are being conducted in Dr. Steven Lindow's laboratory. With this work we are primarily focusing on the interaction between this mutant and the insect vectors during transmission. Is it the lack of bacterial DSF production that makes an *rpfF* mutant not transmissible? And if so, why? Further investigation is needed to answer this important aspect of bacteria- insect-vector interaction.

We also believe that identification of the genes in *Xf* which are responsible for transmission is an essential step to understand vector transmission and bacterial-vector interaction. The identity of these genes may enable us to identify key features of the bacterial mechanism driving transmission. More specifically, this research is seeking to identify the genes regulated by the rpf system and subsequent work should enable understanding of the environmental stimuli affecting them. Better understanding of the required genes and how they interact may lead to new control strategies.

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