EFFECTS OF CHEMICAL MILIEU ON ATTACHMENT, AGGREGATION, BIOFILM FORMATION, AND VECTOR TRANSMISSION OF *XYLELLA FASTIDIOSA* STRAINS

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ABSTRACT
An *rpfF* DSF-deficient mutant that does not adhere to the inside of insect vector mouthparts formed a reduced biofilm *in vitro*, but contributed to biofilm formation when combined with a wild-type *Xylella fastidiosa* (*Xf*)strain. Growth of the DSF-deficient mutant in media PW was comparable to that of the wild type *in vitro* and neither strain appeared to outcompete the other when grown in PW liquid media over two weeks. However the mutant strain showed reduced growth alone, and in co-culture with the wild-type, in a second medium (BHF) that promoted biofilm formation in the wild-type strain. A fluorescent (*gfp*) mutant of Temecula was also used on occasion, but was dropped when it became clear that the mutation reduced growth ability in media and plants. Competitive behavior of the various strains *in planta* is also being investigated in our laboratory. Studies were also undertaken to try to enhance uptake and delivery of *Xf* from artificial feeding sachets to plants. A wild-type and a DSF-overproducing *rpfC* mutant strain of *Xf* were added to sachets and fed to leafhopper vectors. Only one out of 85 insects tested positive for *Xf* after sachet feeding, and no test grape plants have been infected by *Xf* from insects fed this way. Wild-type *Xf* (strain STL), grown on solid media, was also presented for sachet feeding to sharpshooter vectors; one of 24 insects that acquired *Xf* this way was able to transmit to a healthy seedling.

INTRODUCTION
Studies from this lab (R.P.P. Almeida and A.H. Purcell, unpublished) showed that sharpshooters could acquire cultured *Xylella fastidiosa* (*Xf*) cells added to expressed xylem sap in an artificial feeding apparatus (sachet), but did not transmit these bacteria to grapevines, as measured by subsequent symptom development. The assumption was that these cells did not attach to the foregut of the feeding insects. This sort of circumstantial evidence points up the complexity of the bacterial / insect / plant factors necessary for transmission (uptake and delivery) to occur. The importance of attachment and subsequent biofilm formation in this process is also indicated by the behavior of mutants that do not attach to the vector mouthparts and are not insect transmissible (Newman et al. 2004). Studies of *Xf* biofilm formation *in vitro* indicate that chemical make-up of media, substrate, and bacterial genotype/phenotype all play roles in aggregation behavior of *Xf* (e.g., Marques et al. 2002, Leite et al. 2004, Feil et al. 2003, Hoch et al. 2004). Our ultimate objective is to understand the factors that affect the process of attachment and biofilm formation of *Xf* under different environmental conditions. Investigating the conditions, be they environmental or genetic, that promote attachment and subsequent detachment from insect mouthparts is crucial to understanding transmission from insects to plants.

OBJECTIVES
1. Determine whether vector retention (and subsequent delivery) of *Xf* is related to the chemical and physical environment from which the bacteria are grown or acquired.
2. Investigate how *Xf* cells attach (and detach) to specific foregut regions of sharpshooter vectors. *NB: this objective being carried out in collaboration with the Hoch/Burr labs.*

RESULTS
In six experiments in which the *rpfF* mutant KLN61 was grown alone, it made much reduced or no discernible biofilms in glass flasks compared to the wild type Temecula, or the *gfp*-Temecula strain. In an experiment in which biofilms were quantified by sonication of the film after rinsing, and subsequent plating, KLN61 was estimated to make only 6% of the biofilm made by *gfp*-Temecula under identical circumstances. However, both qualitative and quantitative measurement of biofilms created by KLN61 grown together with Temecula (or *gfp*-Temecula) indicated that when the two strains were grown together they were consistently as thick or thicker than those created by Temecula alone.

Furthermore, in four experiments in which we quantified planktonic compared to attached (biofilm) cells of the *rpfF* mutant KLN61 grown together with Temecula or STL wild type *Xf*, the proportion of KLN61 in biofilm was either equal to or greater than the proportion of this strain found in the broth from which the film developed (Table 1). This was determined by sampling broth and a scraping of biofilm from the same flask, and plating on PWG (on which both strains grew) and on selective media (on which only the kanamycin-resistant KLN61 grew). When grown together with the wild-type Temecula, the ability to form biofilm appears to have been restored for the *rpfF* mutant compared to when it was grown alone. The only exception was an early experiment in which we paired KLN61 with the *gfp*-Temecula strain, and in which we found no live KLN61 in the biofilm.

Table 1. Proportion of KLN61 in broth or biofilm in co-cultures with Temecula. Data are from two separate experiments, designated A and B.

	A	Tem+KLN61	KLN61	B	Tem+KLN61	KLN61
Broth- Mean cfu/mL	n=4	1.6×10^8	4.2×10^7 (27%)	n=3	1.4×10^7	2.8×10^6 (21%)
Sample film- Mean cfu	n=7	233.8	57.5 (25%)	n=6	67.2	69.8 (100%)

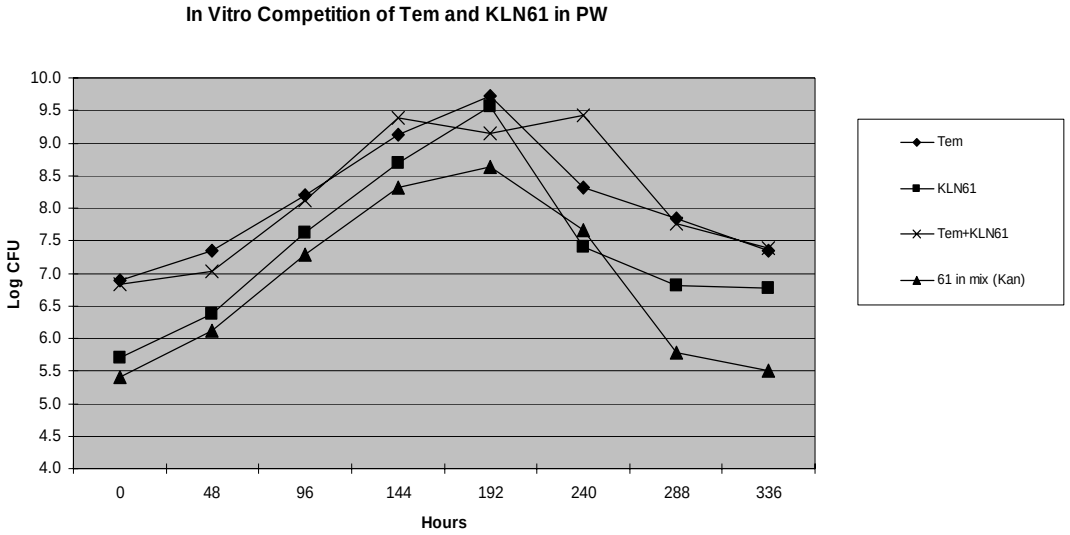

Figure 1. Growth of KLN61 and Temecula strains alone and together in PW broth.

Experiments, done in conjunction with C. Wistrom and C. Baccari in this laboratory, focused on *in vivo* and *in vitro* behavior and population growth of the *rfpF* mutants (KLN61 and KLN62) and the wild-type Temecula strains co-inoculated into grapevines or grown in liquid culture. In the first *in vitro* assay with KLN61 we were not able to determine the proportion of each strain in the co-inoculated media (both strains grew on the "selective media," due probably to an error while making the media). These assays were repeated with PW and a second broth, designated "BHF," to determine whether chemical constituents of the culture medium affect competition potential. BHF is a variant of PW, with added fructose and no BSA that promoted biofilm formation in a wild-type strain of *Xf* (B.H.Feil, unpublished).

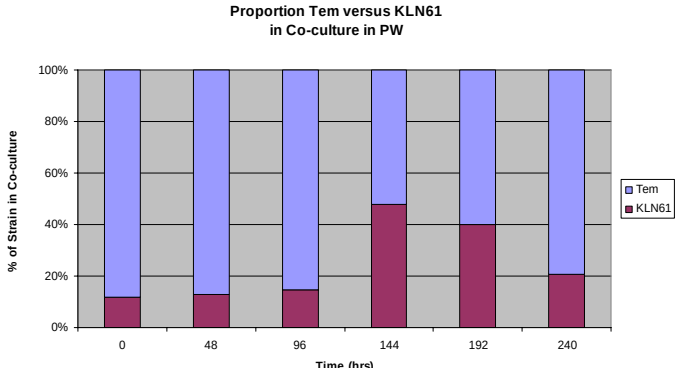

Figure 2. Proportion of the two different *Xf* strains grown together in PW broth.

In PW, growth of the wild-type strain Temecula and KLN61 were comparable (Figure1), though in a previous experiment the population size of Temecula was two orders of magnitude greater than that of KLN61after eight days. When grown together in PW media the proportion of the population made up of KLN61 fluctuated: the proportion approached 50% when the populations were in log phase at 144 hours (even though the initial mixture had a higher proportion of Temecula), and declined as the populations declined (Figure 2).

In one assay using BHF broth, KLN61 did not grow as well as the Temecula strain (Figure 3), and did not fare well in co-culture (Figure 4). Because of variability between assays, additional replicates are needed to determine whether the media (PW vs. BHF) influence the growth and competitiveness of the DSF-deficient compared to the wild-type strain. *In vivo* experiments with these strains are in progress as part of a different project in this laboratory (A.H. Purcell and C.Baccari).

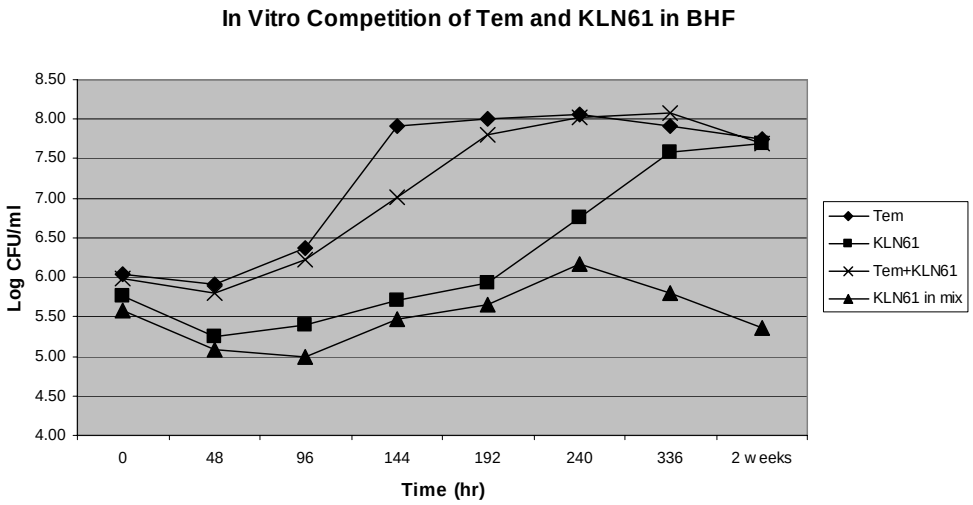

Figure 3. Growth of KLN61 and Temecula strains alone and together in BHF broth.

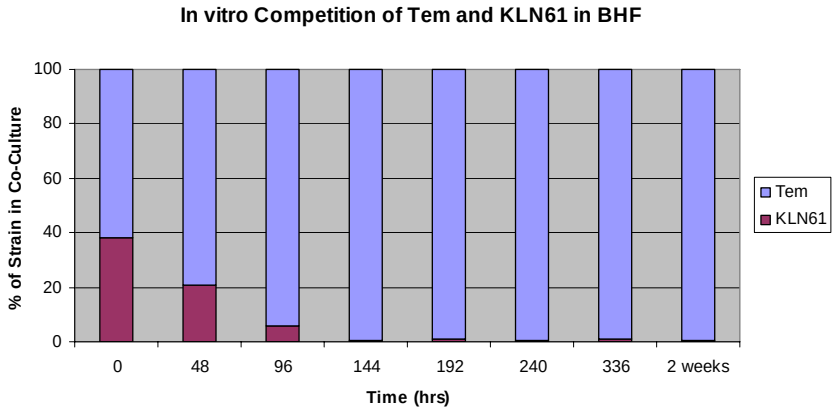

Figure 4. Proportion of the two different *Xf* strains grown together in BHF broth.

We have begun experiments to see how an environmentally-induced phenotype of *Xf* affects acquisition and retention of bacteria by vectors feeding on experimentally manipulated solutions in feeding sachets. Previous work with *Xf* grown on solid media and added to artificial feeding systems showed that vectors acquired but did not transmit bacteria. Several other presentations of *Xf* in sachets were made. *Xf* Temecula strain and a DSF-over-expressing mutant, *rpfC*, from biofilms formed in liquid media were added to sachets of xylem sap; no insects fed on these sachets transmitted to test plants. We have also fed vectors on xylem expressed from symptomatic plants using a pressure bomb or on extracted *Xf* from petioles of symptomatic plants, added to sachets. Heads of these vectors were cultured, but *Xf* was not recovered from them, and insects fed this way did not transmit bacteria to plants. Finally, we fed insects on sachets of *Xf* (strain STL) grown on solid media. One of 24 insects fed this way transmitted to a healthy grapevine. This was encouraging, given that control insects fed on an STL-infected source plant, had very low transmission as well (one of 17 transmitted).

CONCLUSIONS

Our overall objective is to understand the role of aggregation phenomena in acquisition, retention and delivery of *Xf* by vectors. By manipulating the environment in which *Xf* is cultured, we have found differences in the propensity for different strains to form biofilms *in vitro*. The use of *Xf* mutants with impaired or enhanced ability to perform some part of the aggregation behavior will be important to understanding the interaction between environment and bacterial behavior affecting vector retention and delivery. We have been particularly interested in documenting the behavior of *rpfF* mutants and wild-type bacteria alone and together in different liquid media. We have begun to test how some of these same factors affect acquisition and retention of bacteria by vectors feeding on sachets. Our findings may reveal currently unanticipated ways of interfering with vector transmission and elucidate features of *Xf* biofilms applicable to this bacterium in plants.

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

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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).