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

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ABSTRACT
We have measured *in vitro* survival, growth and biofilm formation of wild-type and mutant strains of X*ylella fastidiosa (Xf*) under a variety of conditions. The mutant strains are deficient in their production of the signaling molecule DSF. The role of DSF in biofilm formation is not clear, but these strains cannot attach and/or form biofilms in insect mouthparts (strains KLN 61 or 62, "*rpfF*-mutant"; Newman et al. 2004). Our attachment studies of these strains indicate that the DSF-deficient strain is also less proficient at biofilm formation *in vitro* compared to the *gfp*-Temecula strain. Population studies of wild-type and mutant strains grown separately or together show similar patterns of growth, and no evident competition between strains *in vitro* for up to 14 days. Complementary *in vivo* studies in our laboratory are looking at both strain competition and transmissibility of wild-type and mutant strains of *Xf* co-inoculated into grapevines.

In order to more closely approximate conditions in the insect mouth, we have also assayed attachment of *gfp*-Temecula or wild-type Temecula strain on sterile insect wings, which have a cuticular surface like the interior of the mouthparts to which *Xf* attaches. We have so far not observed bacterial attachment to wings that were incubated in PW broth or in a media deemed more optimal for *in vitro* biofilm formation in preliminary studies. Insect mouthparts and wings (from glassy-winged sharpshooter (GWSS)) are also being investigated as an attachment surface in flow chambers by our collaborators at Cornell University.

INTRODUCTION
Studies from this lab (R. P. P. Almeida and Purcell, unpublished) showed that sharpshooters could acquire cultured *Xf* cells added to expressed xylem sap in an artificial feeding medium (sachet), but subsequently did not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, and plant factors necessary for transmission (uptake and delivery, followed by plant infection) to occur. The importance of attachment and subsequent biofilm formation of the vector transmission process is also suggested by the behavior of mutants that do not aggregate in 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 is similar to one proposed from the Hoch/Burr labs with which we propose to collaborate.

RESULTS
It has been important to optimize growth conditions for *Xf* in order to produce repeatable, accurate assays, and also to begin to determine what environmental factors might be important to manipulate in attachment studies (below). We have tested the effects of pH (5.2-8.0), media (from minimal media through enriched undefined media), vessel (glass, plastic; from 200uL to 30 mL), and source of inoculum (age; from solid or liquid media) on subsequent survival and growth of populations of *Xf* in liquid media. As part of these studies, we have done both short-term survival assays, as well as longer-term population studies. *Xf* populations were measured both by determining absorbance of bacterial suspensions by spectrophotometry (OD_{600}), and by dilution plating of suspensions onto solid media and counting colony forming units per ml (cfu).

Preliminary evidence suggested that xylem sap and a minimal defined medium at a pH of 5.2-5.3 was lethal to *Xf* within 1-24 hours. This was a potentially important finding because it highlighted an area that might be explored as a means of therapy or protection in vines, such as during the dormant season when the vine may be able to best tolerate such acid pHs. Subsequent assays showed that when bacteria were grown under optimal conditions in the laboratory, populations at the lowest pH were slow to grow, but reached population sizes comparable to those at higher pH after six days. This was true for bacteria grown in two quite different liquid media, PW (see Almeida et al. 2004) and CHARD2 (Leite et al. 2004) (data not shown). It is important to note however that the bacteria changed the pH of the media over the course of the assay: after six days, the media that started at pH 5.3 were 6.3 and 5.9 respectively; media of pH 6 became 6.5 and 6.3; and media of pH 7 became 7.2 and 7.1.

The existence of other bacteria is an important part of the chemical and physical milieu in which *Xf* grow, and bears further study. Ongoing studies in our laboratory have pointed to some potentially very interesting interactions between strains of *Xf* in plants. For example, when wild-type strain Temecula and a *gfp* mutant (KLN59.3; Newman et al. 2003) of this same strain were co-inoculated into plants, in no case was both the mutant and the wild-type strain recovered from plants into which they had both been needle inoculated; further, in most cases it was the wild type that persisted (C. Wistrom, unpublished). Researchers in the Lindow laboratory discovered subsequently that the *gfp* gene in this strain had been inserted in a functional area of the genome; hence these results are perhaps not surprising. However, we had also been conducting growth studies of mixed-strain populations of *Xf* in liquid media to see whether we could replicate the competition phenomenon *in vitro*. Experiments with mixed populations of *gfp*-Temecula and wild-type *Xf*, and *gfp*-Temecula and the *rpfF* mutants KLN62 all grown in broth, showed little difference in the growth of the strains separately or together. Populations were sampled at regular intervals, and dilution plated for determination of population sizes. Both *gfp* and *rpfF* mutants can be selectively detected by plating on media containing the antibiotic kanamycin, and the *gfp* mutant can also be detected by epifluorescence microscopy. The proportion of *gfp* bacteria typically dipped but recovered when grown with wild-type or *rpfF* mutant strains (e.g. Figure 1).

Because of the possibility that KLN59.3 (*gfp*) is inherently less competitive, at least *in planta,* we are no longer using this strain in competition experiments. Our experiments with the *gfp* strain have been instructive, nonetheless, as they show that *in vivo* and *in vitro* results can and do differ. All combinations of strains co-inoculated into plants to date indicate that it is rare for two strains to coexist for long periods in plants.

Current experiments, in conjunction with C. Wistrom and C. Baccari in this laboratory, are focusing on *in vivo* and *in vitro* co-inoculations of the *rfpF* mutants (KLN61 and KLN62) and the wild-type Temecula. The first *in vitro* experiment with KLN61 is shown in Figure 2. Although we were not able to determine the proportion of each strain in the co-inoculated broth (due to technical problems), the population size of Temecula was two orders of magnitude greater than that of KLN61 after eight days in PW broth (Figure 2). An earlier experiment with KLN62 showed roughly parallel growth of the two strains when grown separately in PW (not shown). These assays will be repeated, and additional media will be used to determine whether chemical constituents affect competition potential. *In vivo* experiments with these strains are in progress.

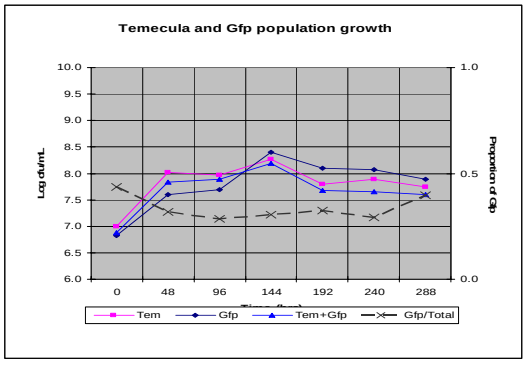

Figure 1. Population growth (log CFU) of Temecula and *gfp*-Tem grown alone and/or together

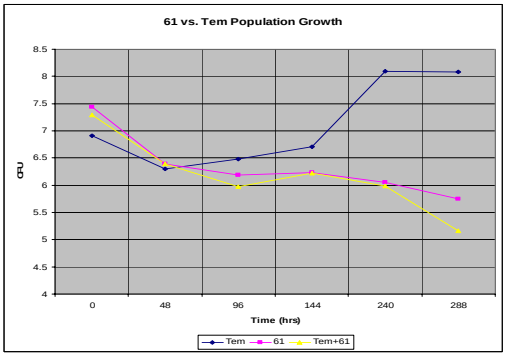

Figure 2. Population growth (log CFU) of KLN61 and Temecula grown alone and/or together

We are interested in the factors that affect attachment and subsequent aggregation behaviors of *Xf*. We have used the information gained on optimization of growth of *Xf* in part to design assays in order to address this question. Using young cultures from plates to inoculate relatively large volumes (30 ml) of liquid PW media, we have examined short-term attachment of 1-3 days, and longer-term aggregation of *Xf* over 7-10 days. We have investigated attachment of *Xf* to vessels of various types using a crystal violet assay (Leite et al. 2004, Espinosa-Urgel et al. 2000). This assay is appropriate for visualization of aggregations of cells *in vitro*, but has not been satisfactory for quantification of attachment in our assays. We

have found that the dye interacts with media and adheres to the substrate without bacteria present, making it difficult to determine whether there are real differences in attachment in the media we are testing.

We also worked with a simpler assay using insect wings glued to glass cover slips, which can be easily manipulated for sterilization, incubation in small amounts of media (with *Xylella*), and mounting on a slide for microscopy. We have carried out these assays with *gfp*-Temecula and wild-type Temecula strain. The *gfp*-Temecula was detected in broth with an epifluorescence microscope, and the wild-type bacteria were stained with DAPI, a fluorescent dye, but were not detected in broth. Neither type of bacteria aggregated on wings in large enough numbers to be detected. We are working to manipulate media, population sizes, and length of incubation to determine which factors will allow for attachment and aggregation. We are also working with dissected mouthparts from the GWSS as a substrate for attachment. Heads and wings of GWSS have been provided to Harvey Hoch as well.

Our interest in the *rpfF* mutants has also extended to our attachment assays. These mutants are deficient in production of the cell-cell signaling molecule DSF (diffusible signal factor), and in biofilm production under certain conditions. In insects they are apparently unable to attach to the mouthparts and form a biofilm there, but in plants mats of bacteria have been seen occluding vessels (Newman et al. 2004). Our attempts to grown biofilms of KLN61 *in vitro* have been occasionally successful, but inconsistent. Our intentions were to compare *in vitro* biofilm production of KLN61 with the wild-type Temecula strain. In order to more easily compare these strains we initially used the *gfp*-Temecula strain (future experiments will use wild-type Temecula because of the problems with the *gfp* strain mentioned above). In two experiments so far we grew the strains separately and together in different media and looked at the proportion of each strain in broth (planktonic cells) compared to in the film itself (a ring of bacteria at the fluid-air interface). In one experiment, KLN61 was not found at all in the biofilm (i.e., the ring consisted of 100% *gfp*-Temecula), even when they made more than half of the planktonic cells (Table 1).

Table 1. Planktonic and biofilm (ring) populations in two liquid media

Medium	Log CFU in broth (*gfp* -Tem plus KLN61)	% Tem-*gfp* broth / ring
BHF	2.1x10^7	45% / 100%
*Xf*D2	1.2x10^7	92% / 100%

In the second experiment, KLN61 was detected in the biofilm, but *gfp*-Temecula made up a disproportionately larger percent of biofilm compared to planktonic cells (25% compared to 16%; Table 2). KLN61 alone also made less biofilm (as quantified by spectrophotometry of dislodged ring cells) than *gfp*-Temecula, in spite of the fact that there were more cells of KLN61 than of *gfp*-Temecula in their respective flasks.

Table 2. Quantification of biofilm production of *gfp*-Temecula and KLN-61 alone and together

	Broth- cfu	% Tem-*gfp* broth / ring	Sonicate (diluted ¼)- OD_{600}
Gfp-Tem	4x10^4	NA	0.065
rpfF (KLN61)	1x10^6	NA	0.004
Both (BHF)	4.3x10^5	16% / 25%	0.053
Both (*Xf*D2)	1.8x10^6		No film

We will continue to explore biofilm production of wild-type *Xf* and *rpfF* mutants to see whether we can find conditions that will change the propensity to form, and the proportions of bacterial strains found, in biofilms *in vitro.*

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 are now ready to see how some of these same factors affect acquisition and retention of bacteria by vectors feeding on sachets. Interfering with vector acquisition and inoculation (reducing or avoiding vector populations) are currently the major control methods for Pierce's disease in California. 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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