A. Project title: Biology of the *Xylella fastidiosa*-vector interface

B. CDFA contract number: 06-0222

C. Time period covered by progress report: November 2007 to March 2008.

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E. List of objectives, and description of activities conducted to accomplish each objective

A. Develop an *in vitro* assay to study attachment of *X. fastidiosa* to sharpshooters in order to determine the nature of molecular interactions between vectors and *X. fastidiosa*.

To accomplish this objective, we have established an *in vitro* attachment assay using foregut extracts and tested several mutants for their adhesion profiles (see previous report).

B. Test the transmission of different *X. fastidiosa* mutants in order to identify proteins implicated in the transmission process.

We have tested if several mutants for different fimbrial and afimbrial adhesins, cell-cell signaling and gum production, were transmitted to plants by sharpshooters.

C. Study the multiplication of *X. fastidiosa* in vectors with mutants affected in transmission and attachment to polysaccharides.

For this objective we used real-time PCR to quantify *X. fastidiosa* mutants and wild-type cells within the head of vectors.

F. Research accomplishments and results for each objective

In our last report we presented results for a series of *in vitro* experiments which demonstrated that *X. fastidiosa* attachment to sharpshooter foregut extracts i) was mediated by proteins on cell surface, ii) had affinity for *N*-acetylglucosamine and similar sugars, and iii) that hemagglutinin-like proteins were important for this process. Here we discuss some of the work since that report.

X. fastidiosa attachment to foregut extracts is correlated with hemagglutinin-like proteins (_hxfA_ and _B_) gene expression levels

Expression level for *hxfA* in the cell-cell signaling mutant *rpfF*- was determined by (Nian et al. 2006). They have showed that *hxfA* was down-regulated in the mutant in comparison with the wild type. Recently, Chatterjee *et al* 2008 showed that *hxfA* was up-regulated in *rpfC*- mutant but down-regulated in the double mutant *rpfF-/rpfC*-. *rpfC*- was determined to be 'sticky' to surfaces, including vectors; while the opposite was observed for *rpfF*-. These results support our previous biochemical findings suggesting that *X. fastidiosa hxfs* have a role in cell attachment to the foregut of vectors (previous report). Accordingly the rare transmission of *rpfF*- (5% in the comparison with wild type) (Newman *et al* 2004) could be explained by the low levels of *hxf* expression in this background. Interestingly, *fimA* and *gumJ* expression in cell-cell signaling mutants have the same expression profile as *hxfA* and *hxfB* (Chatterjee *et al* 2008). Thus, we compared expression levels for these genes in the different mutant backgrounds that were used in our attachment assays, with the objective of determining if *hxfs* gene expression patterns supported observations made with the biochemical work. We found that only *rpfF*-, *rpfF/rpfC*-, *hxfA*- and *hxfB*- mutants have *hxfs* down-regulated compared to the wild type, results which match our biochemical study. In contrast, *hxfA* and *hxfB* were up-regulated in all other mutants tested. Table1 provides a summary of these results.

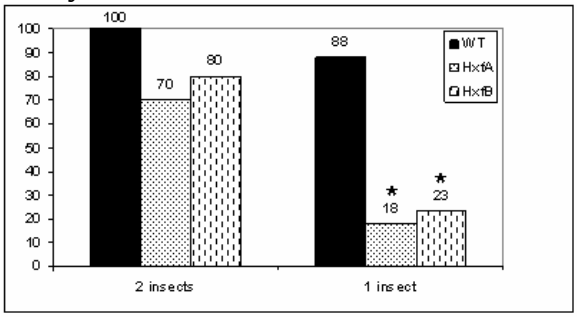
Table1. Relative quantification of gene expression in different mutants by real-time PCR

	Mutant tested / fold change in gene expression ± SE*										
Gene	*Mut1*	*rpfC*	*rpfF*	*rpfF/rpfC*	*hxfA*	*hxfB*	*fimA*	*pilB*	*fimA/pilO*	*gumD*	*gumH*
hxfA	4.1±0.8	2.8±0.3	0.53±0.06	0.8±0.07	-----	0.82±0.03	1.4±0.1	1.2±0.16	1.1±0.03	2.55±0.4	1.68±0.1
hxfB	3.6±0.7	2.11±0.5	0.11±0.03	0.45±0.07	0.9±	----	1.3.±03	1.6±0.2	1.2±0.01	2.2±0.3	1.4±0.1
fimA	n.d.	2.15±0.18	0.4±0.04	0.73±0.19	n.d.	n.d.	----	n.d.	----	n.d.	n.d.
gumJ	n.d.	2.6±0.2	0.56±0.02	0.4±0.04	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* 1.0 = gene mRNA in sample is the same as in the wild type. n.d = not determined.
Shaded boxes from Chatterjee et al. 2008)
Normalization was carried out using 16S ribosomal RNA as an endogenous control.

Vector transmission of *hxf* mutants has low efficiency

Mutants of *hxfA* and *hxfB* were tested for their transmissibility by insects. Transmission tests were performed similarly to those described in Newman *et al* (2004). We found no significant differences *hxfA*- and *hxfB*- mutants and the wild type when groups of two insect were used per grapevine. Significant differences were found when individual insects were used per grapevine. These results indicate that *hxfs* are important for transmission, but are a component of a probably complex interface. Previously we showed that mutants down-regulated for *hxfA* and *hxfB* attached less to foregut extracts *in vitro*. Altogether, we think that the specific binding of *X. fastidiosa* to the



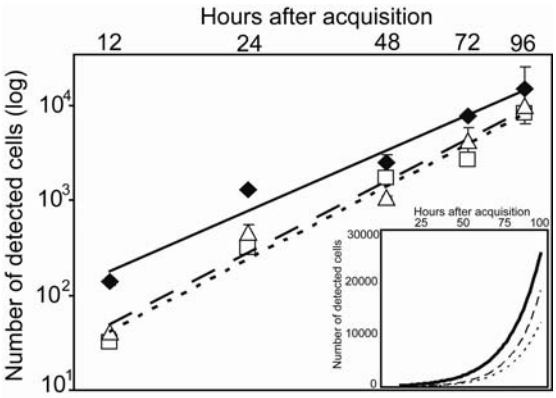
Insect transmission of *hxfs* mutants. Y-axis – transmission rates in experiments conducted.
Transmission tests were performed with 4-day acquisition access periods and 4-day inoculation access periods.
***** Significant difference
2 insects, 1 insect is the number of insects/plant.

carbohydrate-rich foregut of vectors occurs through *hxfs* (important component) and other chitin-binding proteins, and represent the first step of *X. fastidiosa* colonization of sharpshooter vectors.

hxfs have a role in the initial binding of _X. fastidiosa_ to vectors

Although our data suggest that *hxfs* are important for the initial interactions between *X. fastidiosa* and vectors, how can we test that hypothesis? We used a novel approach that proved to be useful for this objective, and will likely be valuable for testing future hypotheses on how *X. fastidiosa* colonizes sharpshooters. If cells are assumed to only be affected in their initial attachment to the cuticle of insect (the case of *hxfs*), quantification of *X. fastidiosa* in the foregut of vectors over time would demonstrate different cell numbers shortly after pathogen acquisition for mutants compared to the wild type. However, as only initial attachment is supposedly affected, cell growth rates after adhesion would be similar for both treatments. We gave sharpshooters a 12-hour acquisition access period on plants infected with the wild type and *hxfA*- and *hxfB*-. Afterwards, insects were confined on basil (poor *X. fastidiosa* host) and randomly collected at various times and cells in the foregut (head) of those individuals quantified. We collected ten insects per time period per mutant (up to 4 days). Eighty percent of insects that fed on grapevines infected with the wild type were PCR positive. Only 38% and 42% were found infected for the insects that fed on plants infected with *hxfA*- and *hxfB*- mutant, respectively.

For positive samples, *X. fastidiosa* populations were determined by real-time PCR (following a protocol described by Francis et al. (2006) and modified by our lab). We also performed a statistical analysis for results obtained from the real-time PCR. For the wild type 12 hours after the acquisition period, we found an average of 138 cells/100ng of head DNA (1 head had on average 300ng of DNA). In contrast, we found ~4 times fewer cells in insects that fed on plants infected with *hxfA*- and *hxfB*- mutants. While the difference between the cell populations in wild type and the mutant treatments was statistically significant in the early stages, there was no significant difference among those 96 hours after acquisition.



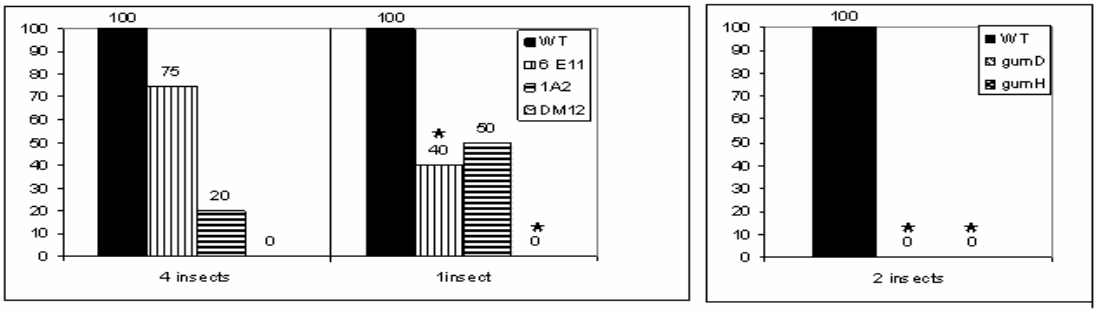
Multiplication of *X. fastidiosa* wild type (solid line), *hxfA*- and *hxfB*- (dashed lines) in sharpshooter's foregut after a 12-hour acquisition access period on infected grapevine. Note differences immediately after acquisition (12h) and slope of lines; results suggest that adhesion to vectors was affected in *hxf* mutants but population growth rate afterwards was similar in all treatments.

Inset – same data, illustrating exponential growth of wild type and mutants in vectors. Growth rate was estimated to be approximately ~2.5 generations/day. r^2 for all exponential curves was higher than 0.95, indicating that up to four days after acquisition cells are multiplying exponentially at equal rates within vectors.

Our results suggest that *hxfA* and *hxfB* are important for initial attachment of *X. fastidiosa* to the cuticle of vectors. Biochemical, gene expression, bacterial colonization and transmission experiments all support that hypothesis. Our data also suggest that i) other polysaccharide-binding proteins on the surface of *X. fastidiosa* contribute to initial adhesion to vectors and ii) *hxfs* may have a role on other steps of biofilm maturation within vectors, but that role may be minimal compared to other surface proteins.

Fimbrial adhesins and gum contribute to transmission

In order to determine if fimbrial adhesins and gum (exopolysaccharides) have a role in transmission biology we also conducted transmission experiments in addition to work previously described. The transmission tests were carried out both with individual insects and groups. Below we show transmission rates for *pilB*-, *fimA*-, *fimA-/pilO*-, *gumD*- and *gumH*- mutants. Interestingly, *gumD*- and *gumH*- mutants were found to be non-transmissible. These mutants can colonize the grapevines but with populations less than the wild type, although their movement in plant seems to be normal. We need to better explore the role of gum in transmission as there are alternative hypotheses to explain our data. *fimA*- (6E11) mutant was significantly affected in its transmission efficiency (40% in comparison with wild type). This mutant was determined to be hypervirulent in plants and move faster than the wild type (Meng et al. 2005). In contrast, *pilB*- (1A2) and *fimA-/pilO*- (DM12) mutants were also affected in their movement in plant because of the absence of pili and were less pathogenic. Thus, at this point it is difficult to determine if these mutants were not transmitted with similar rates as the wild type. These mutants may not have been acquired from plants, or not have attached to vector or colonized insects, and finally, inoculation events may not have generated successful infections. For this reason, we have developed an artificial system to study transmission biology that eliminates variability among mutants in relation to their colonization of plants (more on that in a future report).



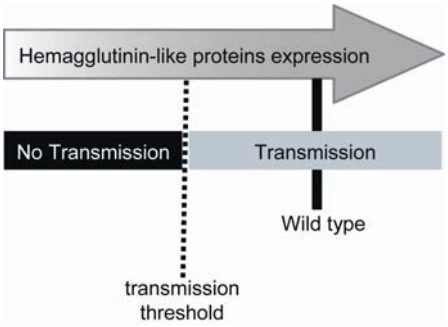
Insect transmission of fimbrial adhesins (left) and gum (right) mutants.
6E11: *fimA*-, 1A2: *pilB*-, DM12: *fimA-/pilO*-

Biology of the *X. fastidiosa*-vector interface – a hypothesis

To summarize the main specific accomplishment of this research project so far: we have developed a hypothesis describing how *X. fastidiosa* cells colonize the sharpshooters. In this model we identify genes contributing in the different stages of biofilm formation. *X. fastidiosa* transmission does not require a latent period, thus, it does not require a mature biofilm. We interpret inoculation events prior to the formation of a mature biofilm as the result of intense turbulence in the precibarium canal due to activity of the sap-pumping system of sharpshooter. In other words, at this stage we believe those events occur primarily by physical, rather than biological, disruption of *X. fastidiosa*-vector interactions. We believe these disruptions are of great importance once a mature biofilm is formed as well and can not be viewed in isolation. However, from a bacterium's perspective, our hypothesis suggests that cells 'prefer' to be sticky prior to biofilm maturation to avoid dislodgment. Spatial heterogeneity exists in biofilms and may explain the early detachment of cells at the center of microcolonies, where conditions mimic those of a mature colony.

Initial attachment

We have shown that *X. fastidiosa* attachment to the foregut of vectors is carbohydrates mediated. *X. fastidiosa* surface membrane proteins act as lectins binding to foregut extracts in vitro. Previously, we showed that attachment to foregut extracts could be reduced in presence of certain sugars, especially *N*-acetylglucosamine. This sugar is the principal unit of chitin and the cement layer of insects, the outermost layer of the cuticle. Reduction in attachment in the presence of sugars indicates that this interaction may be the initial step required for cells to colonize vectors foregut. *hxfA*- and *hxfB*- mutants and mutants affected in their expression for these genes show less attachment to foregut extracts *in vitro*; the same mutants were found to be affected in their transmissibility by insect. To confirm this hypothesis we quantified the acquired cells of *hxfA*- and *hxfB*- mutants in comparison with the wild type. Thus, the expression level of *hxf* proteins may indicate whether a mutant is transmissible or not.



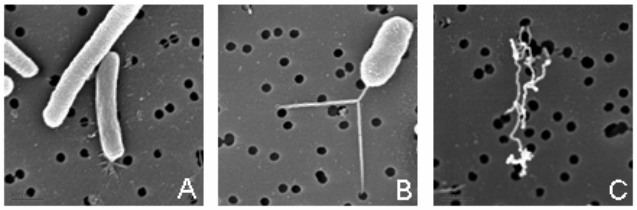
Transmission threshold according to *hxfs* expression levels. In this figure, *hxfA*- and *hxfB*-, mutants are located in the area between the wild-type and transmission threshold. *rpfF*- and *rpfF-/rpfC*- mutants are located in the no transmission area.

An alternative interpretation is that other genes may be important for early *X. fastidiosa* adhesion (e.g. other chitin-binding proteins) and those may be additional components of this model. Our data supports this hypothesis as well. *hxfA* and *hxfB* significantly affect transmission rates, but their absence does not eliminate transmission (area between Wild type and threshold in graphic). Other surface proteins may serve a similar purpose and their multiple elimination can dramatically reduce transmission, such as what happens with the cell-cell signaling mutant *rpfF*-. The lack of availability of a double mutant for both *hxf*s does not allow us to test if those proteins have a redundant function in adhesion to vectors. If the redundancy hypothesis is correct, affecting both proteins would dramatically reduce transmission rates.

Polar attachment and biofilm maturation

Mutant 6E11 (*fimA*-), which retained the peripheral-colony fringe and twitching motility phenotypes, are dramatically affected in its adhering to glass surfaces (Meng et al 2005, Feil et al 2007). The mutant 6E11 is also hypervirulence in plants, probably due to the limited adhesion to xylem vessel walls. Reduced attachment to glass surfaces suggest that attachment through type I pilus adhesins in a non-specific process. Interestingly, *rpfF*-, *hxfA*- and *hxfB*- mutants are also hypervirulent and exhibit reduced vector transmission phenotypes. The *rpfF*- mutant expresses less *fimA* and *hxfs* (Chatterjee et al 2008). This suggests a role for type I pili in biofilm formation in vectors and subsequently in the efficiency of transmission. We showed that *fimA*- mutant expresses more *hxfs* than the wild type; in this context, its reduced transmission rate could be explained by abnormal biofilm formation/maturation in the foregut of vectors. The figure below shows *X. fastidiosa* cells attaching to the surface of a filter membrane. These images confirm the non-specific attachment through type I pili adhesins. The role of long pili (type IV) could be a

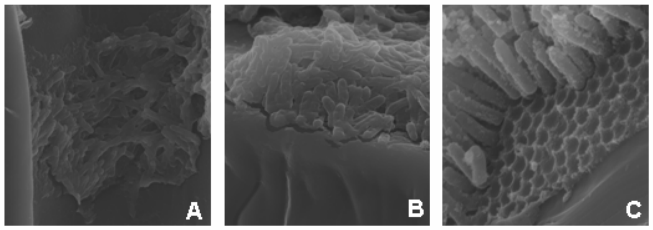
physical role, or assist with the movement of cells during the formation of biofilms in vectors. Note long pili (B) hanging at the pores on the filter.



Scanning electron microscope micrographs for *X. fastidiosa* on filter surface. A: Cells via type I pili. B: Long pili hanging at the filter pores. C: Back of filter showing pili penetrating the pores.

Gum may maintain biofilm structure

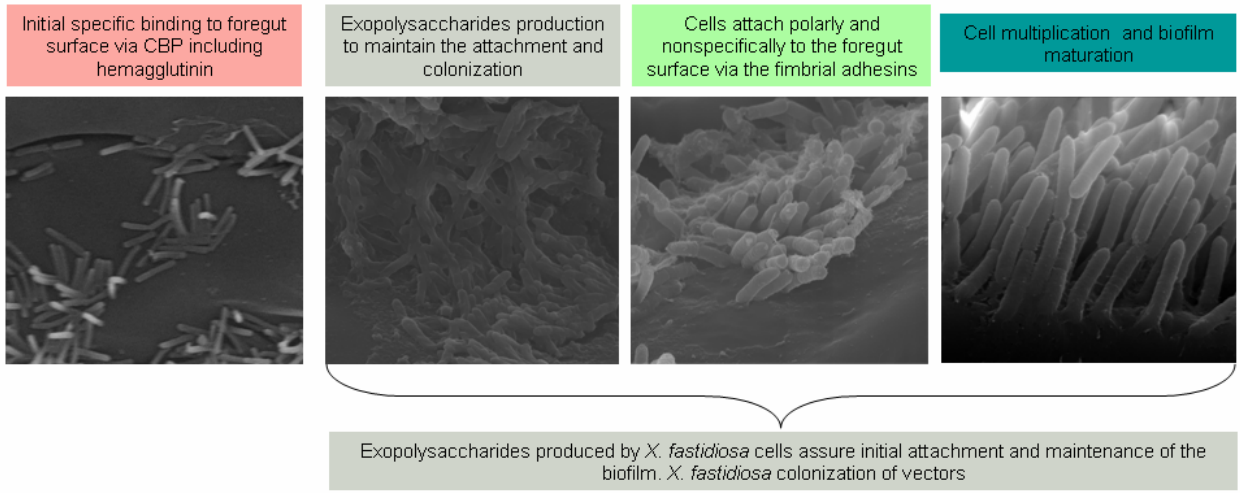
Gum (exopolysaccharides) production play important role in plant virulence by xylem vessels blocking (Roper et al 2007). We have tested two gum mutants for their transmission by insects. Although these mutants express more *hxfs* than the wild type, we found they were not transmissible. Populations of *gumD*- and *gumH*- mutants in plants surveyed were lower than the wild type, thus we are not certain if cells were acquired by insects. For the development of this hypothesis, we assume that gum is important for biofilm maturation as we have always observed it in microscopical studies of *X. fastidiosa* in the foregut of sharpshooters. The figure below shows *X. fastidiosa* cells in the earlier stage after acquisition are embedded in a matrix (Almeida and Purcell 2006). This matrix most likely is gum produced by *X. fastidiosa*. We have also observed a layer of gum coating all cells found in this area of foregut in as microcolonies develop (A and B). In figure C we can see the mature biofilm with cells polarly attached to the cuticle; in this image some cells were released during sample processing. A layer takes the shape of cells was found in the place of cells. At this stage, we believe that the gum cover the entire biofilm, as at earlier stages, but less of it is present (proportionally) and it 'collapses' on the cuticle as the material is dehydrated during processing



Scanning electron microscope micrographs for blue green sharpshooter foreguts infected with *X. fastidiosa*
A: Early stage of colonization (adapted from Almeida and Purcell 2006) B: advanced stage C: Mature biofilm

A hypothesis for *X. fastidiosa*-vector interactions

According to the data collected and our interpretation of them, we developed a hypothesis for how *X. fastidiosa* colonizes vectors. The initial *X. fastidiosa*-vector interaction is mediated by carbohydrate-binding proteins (CBP), including *hxfA* and *hxfB*; in this step cells attach sideways to the cuticle in the foregut of insects. At the second stage cells attach polarly and nonspecifically to the foregut surface via the fimbrial adhesins, eventually forming a mature biofilm. We hypothesize that gum produced by *X. fastidiosa* cells assure initial attachment and maintenance of the biofilm. *X. fastidiosa* colonization of vectors seems to be a stepwise process, much like the formation of a biofilm on solid surfaces.



G. Publications, reports, and presentations where the information generated from the research was presented

Almeida RPP. 2008. *Xylella fastidiosa* ecology. Invited speaker, University of Florida, April.

Almeida RPP. 2008. Vector transmission of *Xylella fastidiosa* – from proteins to models. Invited speaker, University of California, Davis, January.

Almeida RPP, Daugherty MP and Killiny N. 2007. Biology and ecology of *Xylella fastidiosa* transmission to plants. PD/GWSS meeting

Killiny, N. and R. P. P.Almeida. 2007. *In vitro* attachment of *Xylella fastidiosa* to polysaccharides. American Phytopathological Society annual meeting, San Diego, California, USA. 28 july- August 1 (Poster)

Killiny, N. and R. P. P.Almeida. 2007. Biology of *Xylella fastidiosa*-vector interaction. Pierce's Disease Research Symposium, San Diego, CA, Dec. 12-14.

H. Research relevance statement

Vector transmission of *X. fastidiosa* is an essential, albeit neglected, aspect of this system. Until we initiated this project there was no information on how *X. fastidiosa* cells interact with the cuticle of sharpshooter vectors. We have used a multidisciplinary approach to address this question and have demonstrated that initial attachment of cells to vectors, or the first step of sharpshooter colonization, is mediated by carbohydrate-binding proteins on the cell surface. We have also tested *in vitro* and *in vivo* all other *X. fastidiosa* gene mutants available that were assumed to be important for transmission. Results from those experiments suggest that *X. fastidiosa* colonization of vectors is a complex, stepwise process, much like the formation of

biofilms. Together, these results allowed us to develop the first hypothesis on how *X. fastidiosa* interacts with vectors. The hypothesis provides a framework for the community to analyze how cells interact with vectors and, importantly, it provides new opportunities for the development of means to disrupt Pierce's disease spread that are pathogen-vector specific.

I. Summary in lay terms of the specific accomplishments of the research project

The goal of this project was to generate information on how *X. fastidiosa* interacts with sharpshooter vectors at the molecular level. Although this has been assumed to be a complex association, so far it has remained a 'black box'. We have started to dissect this system and our experiments generated information that shone some light into this 'black box'. We now have created a model for the *X. fastidiosa*-vector interactions that can be explored for the development of strategies to disrupt these interactions. For example, our experiments demonstrated that certain sugar-binding proteins on the surface of *X. fastidiosa* cells are important for initial pathogen adhesion to vectors. Now we hope to look for proteins or other compounds with similar binding activity to test if we can saturate these receptors in vectors so that *X. fastidiosa* cells have nowhere to attach in sharpshooters, resulting in no transmission to plants. The determination of how *X. fastidiosa* interacts with vectors will open new venues to control disease spread, as understanding how pathogen and vector interact may lead to strategies to block the transmission of *X. fastidiosa* to plants.

J. Summary of status of intellectual property produced during this research project

None.