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

B. CDFA contract number: 06-0222

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

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

A. Establish 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. We compared the attachment profiles with virulence genes expression levels. We also used GWSS wings as proxies for the foregut surface to study the attachment of GFP-labeled bacteria.

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 (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 the vector foregut 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

***X. fastidiosa* has carbohydrate binding proteins with affinity to *N*-acetylglucosamine, the monomer of chitin**

We presented in the last reports results obtained from several attachment experiments. We showed that *X. fastidiosa* cells have capacity to bind in vitro to polysaccharides and to foregut extracts, and that binding could be repressed by certain sugars. Proteins mediate such adhesion on *X. fastidiosa*'s cell surface. We also found that both *N*-acetylglucosamine and D(+)glucose have a significantly inhibit adhesion (Figure 1-A). Here, we confirm our previous work with new experiments which show the specific binding of *X. fastidiosa* cells to leafhopper

cuticle and that a sugar interferes with cell attachment. We synthesized copolymers from polyacrylamide and one of the sugars D(+)glucose, D(+)galactose and D(+)mannose in the presence of allylamine as a catalyst. The O-glycosylacrylamides were dotted on nitrocellulose membrane in serial dilutions and the membrane blocked with BSA. GFP-labeled *X. fastidiosa* bound to α-D- glucosyl in all dilutions tested, but only to half way with α-D-mannosyl. In contrast, binding to α-D-galactosyl only occurred in dots with little dilution (Figure 1-B). These results strongly suggest *X. fastidiosa* affinity to glucose compared to mannose and galactose. Glucose is the core for the *N*-acetylglucosamine which is the monomer of chitin.

We also conducted another experiments using the GFP-*X. fastidiosa*. In these experiments we used the hindwings of GWSS to mimic the foregut surface. Hindwings have less auto fluorescence than forewings. We found that binding of GFP-labeled cells to wings reduced when we added *N*-acetylglucosamine into the suspension containing cells (Figure 1-C). Interestingly, we found that other plant pathogenic GFP-labeled bacteria did not bind to wings, suggesting the specificity in these interactions (Figure 1-D).

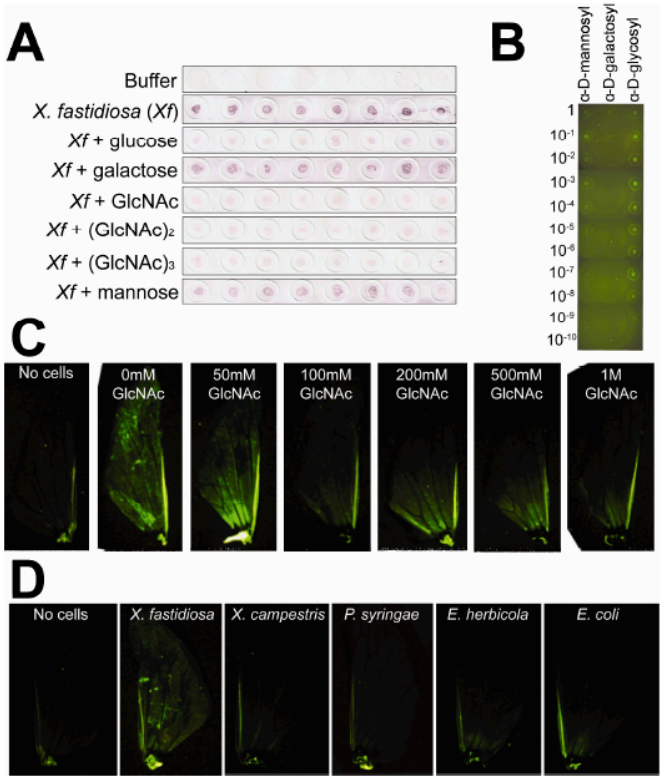

Figure 1: Carbohydrate-mediated inhibition of *X. fastidiosa* cell attachment to surfaces. A) Carbohydrate inhibition of *X. fastidiosa* attachment to leafhopper foregut extracts dotted on nitrocellulose membrane, indicating that cell surface adhesins can be saturated if incubated with certain molecules (GlcNac - *N*-acetylglucosamine). B) Adhesion of GFP-labeled *X. fastidiosa* to carbohydrate-acrylamide copolymers (O-glycosylacrylamides) dilution series. C) Dilution series of *N*-acetylglucosamine inhibiting GFP-labeled *X. fastidiosa* attachment to leafhopper hindwings. D) Specific adhesion *X. fastidiosa* to insect hindwings compared to other plant pathogenic bacteria and *Escherichia coli*.

Lastly, we used HPLC/MS to study the interaction between *N*-acetylglucosamine and *X. fastidiosa* proteins. In this experiment *N*-acetylglucosamine, *X. fastidiosa* cell lysate and *X. fastidiosa* cell lysate treated with *N*-acetylglucosamine were compared. The elution profiles in Figure 2 were compined with the mass spectrums. A shifting of peaks was found in *X. fastidiosa* cell lysate treated with *N*-acetylglucosamine. In addition, a complex was detected in the spectrum. The presence of this complex confirm the specific interaction of *N*-acetylglucosamine with proteins from *X. fastidiosa.*

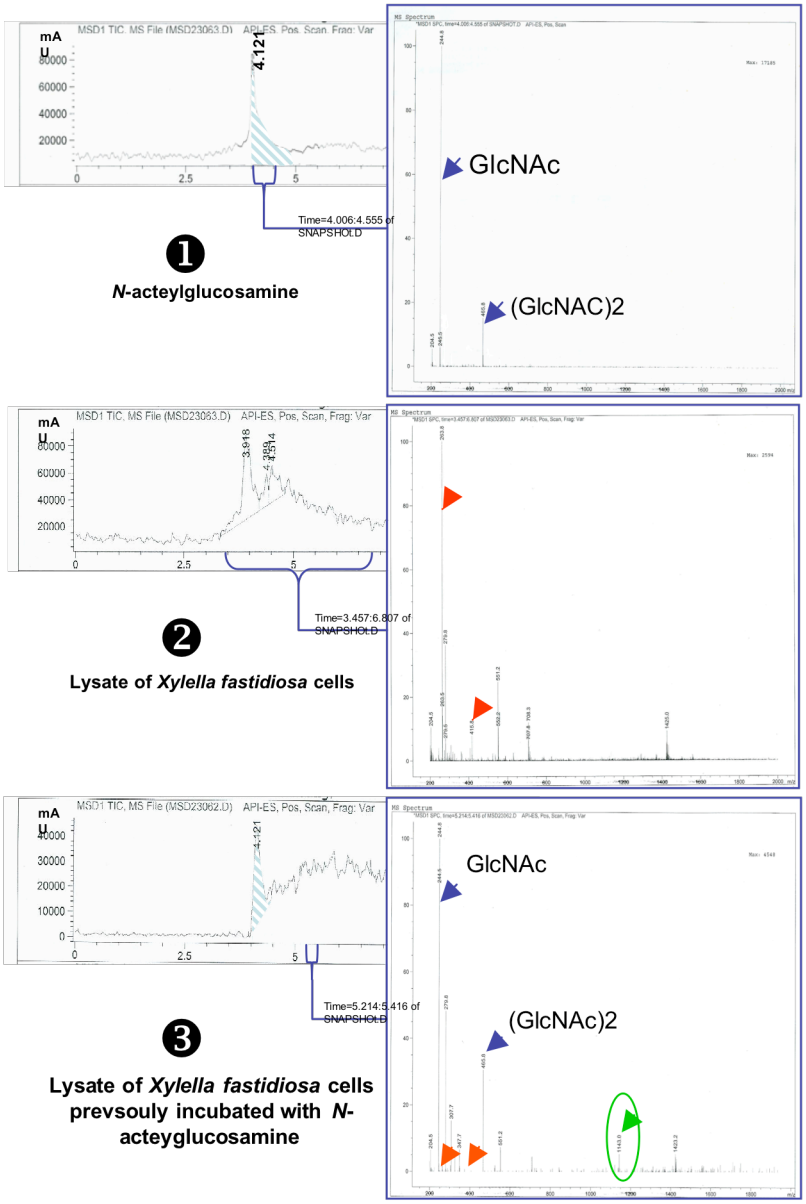

Figure 2: HPLC/MS Analysis for the specific interaction of *X. fastidiosa* cells lysate with the *N*-acetylglucosamine (GlcNAc). The figure shows the elution profile of GlcNAc, *X. fastidiosa* cell lysate and *X. fastidiosa* cell lysate incubated with GlcNAc. The elution profiles are companied with mass spectra of the main peaks, indicating a putative complex of *X. fastidiosa* proteins-GlcNAc at *m/z* 1143. The blue arrows indicate GlcNAc and its dimer (chitobiose) while, the green one shows the complex. The red arrows indicate fragments that disappeared in the *X. fastidiosa* cell lysate profile when incubated with GlcNAc. This fragment is probably implicated in the complex production. Note the shifting of peaks in the elution profile of *X. fastidiosa* cell lysate when mixed with GlcNAc.

Hemmaglutinin-like proteins (HxfA and HxfB) knockout mutants and the mutants affected in their production attach less to polysaccharides and foregut extracts.

In order to identify the proteins which act as carbohydrate-binding proteins we have used a large number of mutant strains in adhesion tests. In this context *hxfA* and *hxfB* mutants showed less attachment to foregut extracts and other polysaccharides. In addition the cell-cell signaling mutants *rpfF*- and *rpfF-/rpfC*- also showed less attachment (figure 3-A). We studied the expression profile of virulence genes in all mutants used in this study including those which were not affected in their adhesion. The expression tests showed that both *rpfF*- and *rpfF-/rpfC*- have a down-regulated profile for *hxfA* and *hxfB*, in contrast the level of *hxf* expression in the other mutants was normal or even up-regulated in comparison with the wild type (figure 3-B). Interestingly, the mutants of *rpfF*- and *rpfF-/rpfC*- are dramatically affected in their transmission by BGSS (Newman et al 2004 and our findings). This defect in transmission could be explained

by the lack of the optimal quantities of Hxf in these mutants. These findings encouraged us the study the transmission of Hxf mutants and their growth in vectors. A plant hyper-virulent phenotype was found for the knockout mutant *hxfA*- and *hxfB*- (Guilhabert and Kirkpatrick 2005). This phenotype was explained by the lack of attachment in xylem and fast movement in plant. In contrast, for vector transmission cells need to attach to a surface. Thus, we hypothesized that the *hxf* mutants were affected in initial adhesion to vectors. In order to test this hypothesis, we conduct the experiments described below.

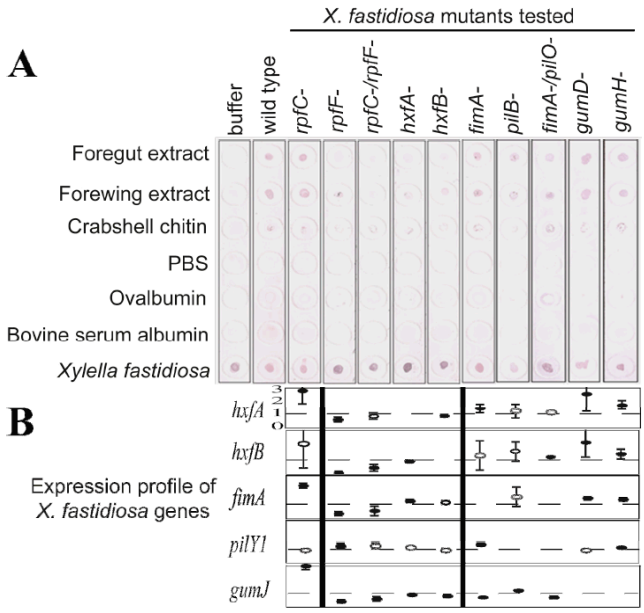

Figure 3: A) Attachment of *X. fastidiosa* knockout mutants to nitrocellulose membranes coated with vector foregut extracts, and other substrates. B) Real-time PCR based quantification of gene expression profile (5 genes, rows) for all mutants used in the biochemical adhesion assay in comparison to the wild type. Dashed line shows expression level in relation the wild type; values larger than one indicate overexpression and values smaller than one indicate repression. Solid circles show values statistically different from the wild type and empty circles those equal to the control; standard error indicated by vertical lines. Y-axis represents the fold-change in gene expression.

The early colonization of insect foregut by *X. fastidiosa* is mediated by Hxf and potentially other carbohydrate-binding proteins.

According to the previous results, we conducted transmission tests with *hxf* mutants. Both *hxfA*- and *hxfB*- mutants were less transmissible than the wild type. Although our data suggest that *hxfs* are important for the initial interactions between *X. fastidiosa* and vectors, how could 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 hypothesized 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) (Fig. 4). 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.

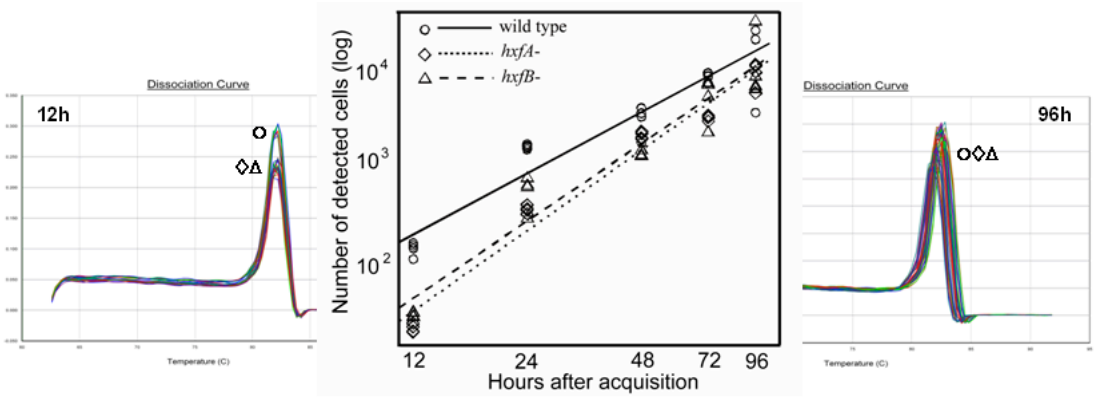

Figure 4: Bacterial populations within leafhopper vectors over time after a 12-hour pathogen acquisition access period. Wild type (solid regression line), *hxfA*- (dotted regression line) and *hxfB*- (dashed regression lines). Note values immediately after acquisition (12-hour period) and 4 days afterwards. Fewer *hxfA*- and *hxfB*- cells adhered to vectors, but after a few days populations were of equal size.

The exopolysaccharides and type I pili (short pili) are involved in the transmission process.

We also tested if the interaction between *X. fastidiosa* and vectors are only carbohydrate mediated or more complex. For that, we used other mutants of intereste for their transmission by BGSS, including fimbrial adhesins and gum (exopolysaccharides). We conducted transmission experiments in addition to the 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.

In addition to these strains, we conducted transmission tests with a new cell-cell signaling mutant (cyclic-di-GMP signaling system). This mutant shows hyper-attachment to surfaces and produces expolysaccharides 4 times more than the wild type. Scanning and transmission electron

microscopy showed that cells are attached together in aggregates and covered by large quantities of extracellular matrix (data non shown). The mutant is transmitted with rate 30% in comparison with the wild type. The cell-cell signaling *rpfC*- is also affected in its transmission rates and exhibits similar phenotype as the GDEEF mutant (Chatterjee et al. 2008). Thus, we think that these mutants have a hyper-attachment to foregut surfaces which makes releasing cells more difficult, reducing transmission rates. The cell-cell signaling mutants have a different gene expression profile than the wild type, thus the role of the targeted genes in these mutants are indirect.

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

To summarize the specific accomplishments of this research project so far, we have developed a hypothesis describing how *X. fastidiosa* cells colonize 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 cannot 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.

We have shown that *X. fastidiosa* attachment to the foregut of vectors is carbohydrate mediated. *X. fastidiosa* surface membrane proteins act as lectins binding to foregut extracts in vitro. 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. *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 insects. Another interpretation of our results 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. Other surface proteins may serve a similar purpose and their multiple knockout may dramatically reduce transmission rates, 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, eliminating both proteins would dramatically reduce transmission rates.

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 hyper-virulent in plants, probably due to the limited adhesion to xylem vessel walls. Reduced attachment to glass surfaces suggests that adhesion through type I pili is non-specific. Interestingly, *rpfF-, hxfA*- and *hxfB*- mutants are also hyper-virulent 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 role of long pili (type IV) could be a physical, or assist with the movement of cells during the formation of biofilms in vectors. Gum (exopolysaccharide) production plays 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 insects acquired cells. In 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.

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

Killiny, N. and Almeida, RPP. 2008. *Xylella fastidiosa* afimbrial adhesins mediate cell attachment to leafhopper vectors and transmission to plants. *Submitted.*

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, describing how this research will contribute towards solving the PD/GWSS problem in California

The interactions between *X. fastidiosa* and its vectors represent an untapped source of PD spread control alternatives. We are exploring the interactions among these organisms in search of approaches to reduce pathogen spread within vineyards. Through our studies we now have a better understanding of how cells bind to vectors. Using that knowledge, we have been able to block *X. fastidiosa* adhesion to the cuticle of vectors in vitro. We are now trying to develop approaches to block *X. fastidiosa* transmission from plant to plant by providing leafhoppers with compounds that may reduce cell adhesion and consequently transmission.

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

This research project's main objective was to understand how *X. fastidiosa* colonizes sharpshooter vectors. We used a series of approaches to demonstrate that *X. fastidiosa* cells have sugar-binding proteins on their surface that bind to leafhoppers. We identified these proteins out of a series of putative candidate, demonstrating biochemically, molecularly and through bioassays that two specific proteins are important for *X. fastidiosa*'s early colonization of vectors. We hypothesized and demonstrated that, by disrupting these interactions in vitro, *X. fastidiosa* adhered less to the cuticle of vectors.

J. Summary and status of intellectual property produced during this research project
None produced.