BIOLOGY OF THE *XYLELLA FASTIDIOSA*-VECTOR INTERFACE

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ABSTRACT
The Gram-negative bacterium *Xylella fastidiosa* (*Xf*) is the causal agent of many economically important plant diseases. This bacterium attaches to, multiplies in, and is inoculated from the foregut of sharpshooter leafhoppers, which feed on plant xylem sap. Little is known about the specific interactions between *Xf* cells and its vectors' foregut. Constituents of the foregut include mucopolysaccharides, chitin, proteins and other components. We have developed an in vitro assay to study the attachment of *Xf* to carbohydrates using nitrocellulose membranes coated with different polysaccharides. We found that *Xf* cells attach to polysaccharides that contain a chain of glucose or *N*-acetylglucosamine. These results suggest that the attachment of *Xf* cells to surfaces may be carbohydrate mediated. *Xf* cells treated with either protease K or pronase showed less attachment to the polysaccharides tested. Competition experiments with different sugars and lectins suggest that attachment of *Xf* to vector's foregut is carbohydrate mediated and that proteins of *Xf* may work as lectins that have affinity to sugars, especially glucose and *N*-acetylglucosamine. In order to identify carbohydrate binding proteins in *Xf*, we have studied the attachment of 15 different mutants to foregut extracts and different polysaccharides blotted in nitrocellulose membranes. Only hemagglutinin-like protein mutants exhibited less attachment. Insect transmission tests for the different mutants were also done; we found a correlation between the in vitro attachment assays and vector transmission to plants of the mutants tested.

INTRODUCTION
Sharpshooter transmission of *Xf*
Transmission of *Xylella fastidiosa (Xf)* by sharpshooters is unique in terms of vector-pathogen relationships. Firstly, the interactions seem to be group-specific when compared to other systems, i.e. virtually all xylem sap-sucking insects can (and those tested do) transmit different strains of *Xf* (Almeida et al. 2005). Such lack of specificity suggests that there is a general mechanism for pathogen attachment, multiplication and detachment. The lack of transstadial transmission and latent period indicates that the *Xf* inoculum is limited to the cuticle of the foregut of vectors, which is shed with each nymphal molt. Purcell et al. (1979) and Brlansky et al. (1983) showed the presence of *Xf* in the cibarium and precibarium of vectors, corroborating the assumption that cells should be present in the foregut of infective individuals. Newman et al. (2003), using cells with a GFP-*Xf* cells, demonstrated the presence of cells in the precibarium of infective vectors. The direct association of the precibarium as the source of inoculum, however, has only been recently demonstrated. Newman et al. (2004) showed that poorly-transmitted *Xf* mutants did not colonize the precibarium of vectors; and Almeida and Purcell (2006) showed that insects transmitting *Xf* had the bacterium in their precibarium, whereas non-transmitting individuals did not. The later work indicated that *Xf* must attach to the precibarium of vectors to be inoculated into plants.

Molecular interactions between *Xf* cells and the foregut of vectors
Xf cells probably have complex interactions with the foregut of vectors, as other xylem-limited bacteria such as *Leifsonia xyli* are acquired but not transmitted by insects (Barbehenn and Purcell 1993). However, little is known about the specific interactions between *Xf* and the foregut of vectors. On the other hand, the insect's cuticle structure is reasonably well understood. The cuticle lining of the foregut of sharpshooters, to our knowledge, has not been studied, but is assumed to be similar to the cuticle of insects in general. Considering the components of cuticle, we hypothesize that *Xf*'s attachment to cuticle could be mediated by carbohydrates. Those could be mucopolysaccharides, glycoproteins, chitin or other polysaccharides and lipopolysaccharides. Thus, our approach was to initially determine the general type of interaction between *Xf* and components likely to be present on the surface it attaches to in insects.

OBJECTIVES
1. Determine the nature of molecular interaction between sharpshooter vectors and *Xf*.
2. Develop an *in vitro* assay to study attachment of *Xf* to sharpshooter foregut and polysaccharides.
3. Identifying *Xf* proteins involved the transmission process.

RESULTS
I- Characterization of *Xf*-vector interface
An in vitro assay was developed to study the attachment of *Xf* cells to polysaccharides. In this method, pieces of nitrocellulose membrane were soaked for one hour in different polysaccharide solutions (concentration 1%), which were

washed and incubated with buffer or *Xf* cells. Detection of cells was done with Ponceau S or amido black dyes. Both of them are protein specific dyes, but amido black is more stable and quantifiable.

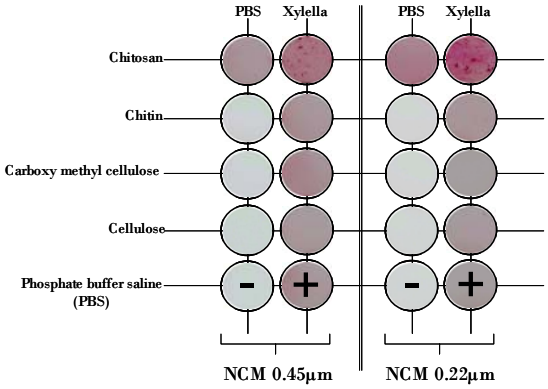

Figure 1. Attachment of *Xf* cells to different polysaccharides. Attached cells were detected by protein specific staining using Ponceau S. (darker spots indicate presence of *Xf*). The PBS columns are a buffer control. Note that chitosan stained unspecifically, but *Xf* cells were still detectable. Some of these polysaccharides are expected to be similar to those present in the foregut cuticle of vectors.

Figure 2 shows results obtained from tests performed with wheat germ agglutinin (WGA) as a lectin and amido black as protein staining dye. In this experiment attachment of WGA to polysaccharides was used as a positive control and to measure the binding of the *Xf* cells to polysaccharides. *Xf* cells showed an attachment profile similar to that of the lectin WGA. In contrast, bovine serum albumin (BSA) negative controls did not attach to any polysaccharide except chitosan, with which we have consistently found background problems. This experiment showed that the attachment to polysaccharides is specific and confirms that *Xf* cells have outer membrane molecules which act as lectins and attach to polysaccharides.

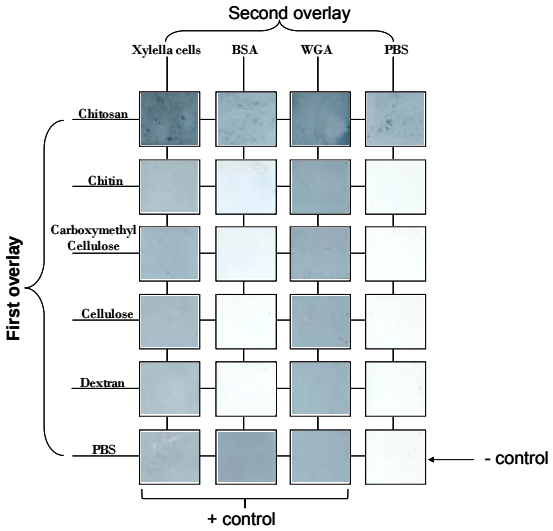

Figure 2. *Xf* attachment to polysaccharides. Acronym: *PBS: Phosphate buffer saline.*

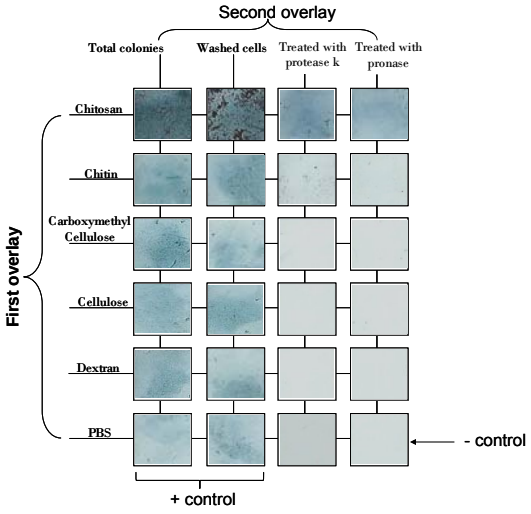

Figure 3. Effect of protease treatment on *Xf* attachment to polysaccharides.

We also studied the effect of treatment of *Xf* cells with proteases on its attachment to polysaccharides (Figure 3). The aim of the experiment was to determine if secreted and membrane proteins play a role in attachment. Washed cells showed similar attachment to polysaccharides as the control. On the other hand proteinase treated cells attached less to the polysaccharides. These results suggest that *Xf* secreted proteins may play a minor role in attachment and that membrane proteins have an important role in this process.

II- Identification of *Xf* membrane proteins associated with attachment to polysaccharides *in vitro*

In order to identify carbohydrate binding proteins in *Xf*, we used several mutants including those of cell-cell signaling and hemagglutinin-like proteins, pili and gum mutants. Hemagglutinin genes affect the virulence of *Xf* in grapevines. The mutants *hxfA*- and *hxfB*- showed hypervirulence in plants; and interestingly attached less to glass than the wild type (Guilhabert and Kirkpatrick 2005). The series of *rpf* cell-cell signaling system mutants have different attachment to glass, aggregation and biofilm formation phenotypes (Newman 2004, S. Chatterjee *personal communication*). In addition, we also used new mutants that were produced by the Lindow lab, which targeted *Xf*'s intracellular cell-cell signaling system; in our study those mutants were labeled mut1 and mut2 (the difference is the mutation site in the gene). Both mut1 and mut2 attached to glass and formed more cell aggregates than the wild type. We also used another *hxfB* mutant, produced by

homologous recombination (Feil *et al*. 2007). Pili and fimbriae mutants were thought to be important in the attachment to foregut and the insect transmission and were included in the study. Bacterial gum production plays a role in virulence in plant like host xylem vessel blockage (Roper et al 2007). As the role of gum in the interactions with vector is unknown, we included two gum mutants in our study. *In vitro* attachment assays were performed to test these mutants in their capacity to bind to foregut extracts and polysaccharides. In these assays, foregut extracts, foregut proteins (extracted by ultra sonication and acetone precipitation), wing extracts, crab shell chitin, PBS, ovalbumine and BSA were dotted in strips of nitrocellulose membrane and *Xf* cells added as an internal control in each strip. After blocking with 6% non fatty milk in PBS (0.1M), strips were incubated with PBS containing *Xf* mutants. The interaction buffer had 0.01M PMSF as anti-protease to protect cells from degradation. Immunological detection of attached *Xf* cells was performed using purified IgGs polyclonal antibodies against *Xf* whole cells.

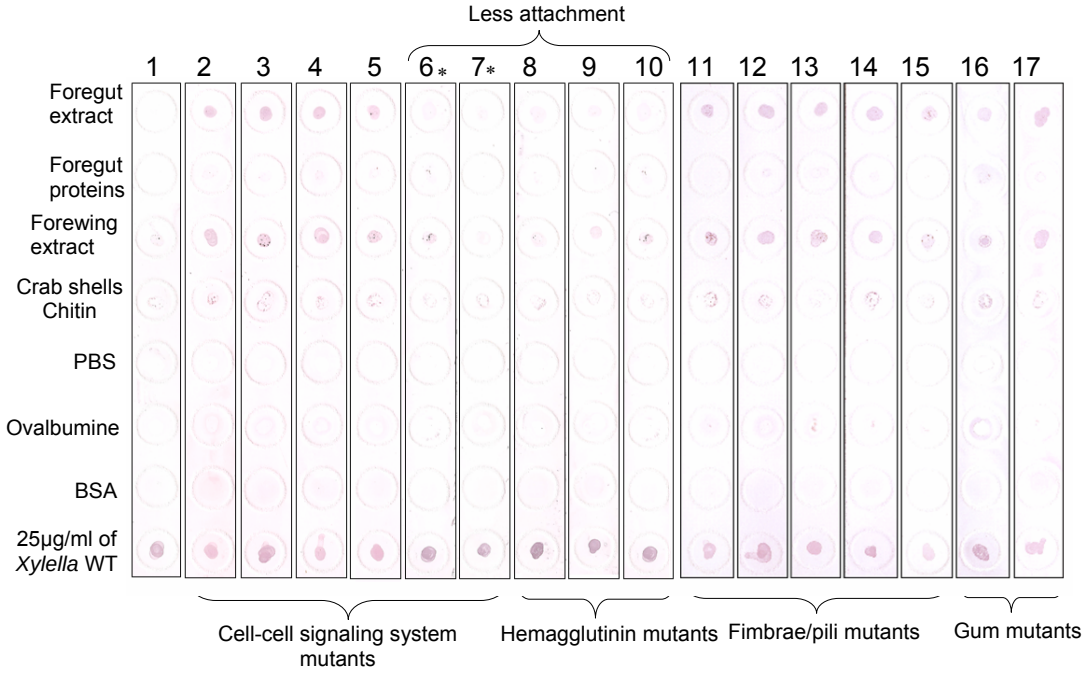

Figure 4. Attachment of *Xf* mutants to foregut extracts and polysaccharides *in vitro*. 1: PBS, 2: WT, 3: mut1, 4: mut2, 5: rpfC, 6: rpfF, 7: rpfF/rpfC, 8: hxfA, 9: hxfB, 10: hxfB (Feil), 11: fimA, 12: fimF, 13: pilB, 14: fimA, (6E11), 15: fimA/pilO (DM12), 16: GumD, 17: GumH. Strips were incubated with 2ml of 50µg/ml of *Xf* cells. Immunodetection performed with polyclonal antibodies against whole *Xf* cell followed with anti-rabbit IgG (Whole molecule) - Alkaline phosphatase antibodies. Alkaline phosphatase activity was detected by BCIP/NBT substrate.

Results obtained from this experiment show that hemagglutinin-like protein mutants are affected in the attachment to polysaccharides and foregut extracts. In addition, only the mutant *rpfF* and the double mutant *rpfF/rpfC* were also affected in attachment to the compounds tested. In studies on the gene expression of *HxfA* in the *rpfF* mutant, Lindow`s group found that *hxfA* was expressed less in the mutant in comparison to the wild type (Nian et al 2006). These results suggest an important role for hemagglutinin in *Xf* attachment to the foregut of sharpshooters. Also the low expression of hemagglutinin in the *rpfF*- mutant could explain the low percentage of its insect transmission (5% in the comparison with wild type) (Newman et al. 2004).

III- Sugar affinity

Competition experiments with different sugars were carried out in order to determine the affinity of the carbohydrate binding proteins in *Xf*. Results obtained showed that the presence of glucose, N-acetylglucosamine (GlcNAc), chitobiose ($[GlcNAc]_2$), and chitotriose($[GlcNAc]_3$) reduced the attachment of *Xf* to foregut extracts. Mannose also had an effect in reducing the attachment but no effect was observed for galactose. This affinity suggests the presence of chitin binding proteins (ChBPs) in the *Xf* cell membrane. These ChBPs could play an important role in the attachment to foregut and the efficient transmission of *Xf* to plants.

VI- Vector transmission of *Xf* mutants to plants

We conducted several experiments to determine the transmission efficiency of *Xf* mutants to plants in relation to the wild type. In the first one, we studied the transmission and retention of *rpfB*- mutant. We observed that *rpfB*- mutants were

transmitted similarly to the wild type, but that cells were not observed in the foregut of vectors by scanning electron microscopy. Instead, a complex matrix was detected (Almeida and Lindow, unpublished data). We hypothesized that *rpfB*- could be lost by vectors over time, and that transmission efficiency would be reduced with sequential transfers of infective vectors to new plants. This was confirmed with transmission experiments. In another experiment, we looked at the transmission of fimbrial adhesin mutants (*fimA*-, *pilB*- and a *fimA-/pilQ* double mutant). Transmission experiments showed that these appendages are not essential for vector transmission. The mutants *fimA*- and *pilB*- were transmitted less efficiently than the wild type, and the *fimA-/pilO*- double mutant was not transmitted, but we were not able to determine if that is due to the *Xf*-vector interaction or low pathogen population in plants that may have affected acquisition efficiency. S. Lindow, a cooperator in this project, performed work with an *rpfC*- mutant. *rpf* genes are associated with *Xf*'s cell-cell signaling and have been shown to affect pathogenicity and transmission to plants. We have transmission data for three *rpf* mutants (*F, C, B*). *rpfF*- is poorly transmissible, *rpfC*- has transmission rates slightly lower than the wild type and *rpfB*- is poorly retained by vectors. For a few of these mutants it is difficult to determine if they were not transmitted with similar rates as the wild type because i) they were not acquired as well, ii) they did not attach to vector's cuticle, iii) acquisition and retention occurred, but inoculation events did not generate infections, or iv) those successful infections did not multiply/move within plants and were not detected. Nevertheless, the results show that fimbrial adhesins are not essential for *Xf* transmission and that different genes are likely important for cell attachment, indicating that this is a complex biological process. As a side note, our results also demonstrated one more time how inefficient glassy-winged sharpshooter is in transmitting *Xf* to plants when compared with Blue-green Sharpshooter (data not shown). We are currently conducting tests with other mutants of interest identified by our in vitro work. In addition, gene expression data of several mutants and their analysis in relation to transmission biology indicate that hemagglutinin expression is strongly correlated with vector transmission of *Xf* to plants. On the other hand we showed that, fimbrial adhesions are not implicated in insect transmission. Taken together, our results suggest that hemagglutinin-like proteins are important for efficient vector transmission of *Xf*. Due to the biological properties of hemagglutinin, much of our current work is focused on glycobiology.

CONCLUSIONS

The goal of this project was to generate information on how *Xf* interacts with leafhopper 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. We demonstrated that fimbrial adhesins, which were previously considered as essential for transmission, are not required for it. In addition, our biochemical characterization of these interactions indicated that proteins are associated with attachment to vector's cuticle and tentatively identified hemagglutinins as important for *Xf* transmission. We are now testing if that [hemagglutinin] gene (two copies in the Temecula genome) is essential for transmission. In summary, we went from a 'black box' to testable hypotheses and the identification of genes that may be important for *Xf* vector transmission. The determination of how *Xf* 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 *Xf* to plants.

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