BIOLOGY OF THE *XYLELLA FASTIDIOSA*-VECTOR INTERFACE

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ABSTRACT
The interactions between the economically important plant pathogenic bacterium *Xylella fastidiosa* (*Xf*) and its leafhopper vectors have been poorly characterized. We used different approaches to determine how *Xf* cells interact with the cuticular surface of the foregut of vectors. We demonstrate that *Xf* binds to different polysaccharides with variable affinities, and that these interactions are mediated by cell surface carbohydrate-binding proteins. In addition, competition assays showed that N-acetylglucosamine inhibited bacterial adhesion to vector foregut extracts and intact wings, demonstrating that attachment to leafhopper surfaces can be affected in the presence of specific polysaccharides. In vitro experiments with several *Xf* knockout mutants indicated that hemagglutinin-like proteins were associated with cell adhesion to polysaccharides. These results were confirmed with biological experiments, when hemagglutinin-like proteins mutants were transmitted to plants at lower rates when compared to the wild type. Furthermore, although these mutants were defective in adhesion to the cuticle of vectors, their growth rate once attached to leafhoppers was similar to the wild type, suggesting that these proteins are important for *Xf* initial adhesion to leafhoppers. We propose that *Xf* colonization of leafhopper vectors is a complex, stepwise process, similar to the formation of biofilms on surfaces. Results presented here and in the 2007 report have been combined and submitted to publication.

INTRODUCTION
The interaction of *Xylella fastidiosa* (*Xf*) with the foregut cuticle differ from other xylem-limited bacteria such as *Leifsonia xyli* which can be acquired from plants but are not transmitted by insects (Barbehenn and Purcell 1993). Only two studies with *Xf* knockout mutants have addressed aspects of vector transmission (Chatterjee et al. 2008, Newman et al. 2004). However, both studies focused on *Xf*'s cell-cell signaling system, which regulates cascades of genes and pathways, thus allowing the identification of target genes, but not identifying specific interactions between vector and pathogen. The *rpfF* gene (Regulation of Pathogenicity Factors *F*) encodes an enzyme that synthesizes the signaling molecule DSF (diffusible signaling factor), whereas *rpfC* is part of a hybrid two-component DSF sensor (Chatterjee et al. 2008). An *rpfF*- mutant is not transmissible by insects because it does not colonize the foregut of vectors (Newman et al. 2004), while *rpfC*- colonizes insect's foregut but is transmitted at lower rates compared to the wild type (Chatterjee et al. 2008). In vitro adhesion assays indicated that *rpfF*- did not form biofilms, while *rpfC*- adhered to surfaces more strongly than did the wild type. Targeted gene expression analyses of *Xf* adhesins indicated that hemagglutinin-like proteins (afimbrial adhesins) and type I pili were associated with adhesion and transmission of these knockout strains, but type IV pili were not (Killiny and Almeida submitted). Thus, indirect evidence allowed us to hypothesize that some adhesins are important for *Xf* attachment to and colonization of vectors, and subsequent inoculation into susceptible hosts, while other adhesins are putatively of little or no role in this process. In this study we sought to determine the nature of *Xf*-vector interactions using biochemical, molecular and biological assays.

OBJECTIVES
1. Determine the nature of the *Xf*-vector interactions.
2. Identify *Xf* surface proteins involved in the transmission process.
3. Develop an artificial diet system to study *Xf* transmission.
4. Identify molecules that disrupt *Xf* adhesion to vectors.

Here we report on the first two objectives mentioned above.

RESULTS AND DISCUSSION
N-acetylglucosamine blocks *Xf* adhesion to insect surfaces.
To determine the affinity of *Xf* cells to different sugars we used a competition assay based on the concept that polysaccharide-binding proteins on the surface of *Xf* can be saturated by exogenous molecules, reducing overall cell attachment to leafhopper foregut extracts. D(+)-galactose did not interfere with the binding of *Xf* to foregut extracts while D(+)-mannose had a small effect (**Figure 1A**). However the monomeric moiety of chitin, *N*-acteylglucosamine, as well as its dimmer chitobiose and its trimer chitotriose, as well as its core molecule glucose, blocked cell adhesion to leafhopper foregut extracts (**Figure 1A**). Thus, specific carbohydrates inhibit *Xf* adhesion to extracts from leafhopper vectors. The affinity of *Xf* carbohydrate-binding proteins to sugars was also tested using synthetic copolymers as described by (Chadli et al. 1992). Our goal was to eliminate potential sources of error on the competition assays, as the previous experiment was performed using

leafhopper extracts mimicking *in vivo* conditions that could have other factors affecting the tests. We also used GFP (green fluorescent protein)-labeled *Xf* (Newman et al. 2003) to limit sample processing. We determined that cells specifically bound to the glucosyl ligand "poly (O-α-D-glucopyranosylacrylamide) copolymer". A negligible interaction was obtained with the galactosyl ligand, while binding of *Xf* to the mannosylated copolymer was detected half way through the dilution series used (**Figure 1B**).

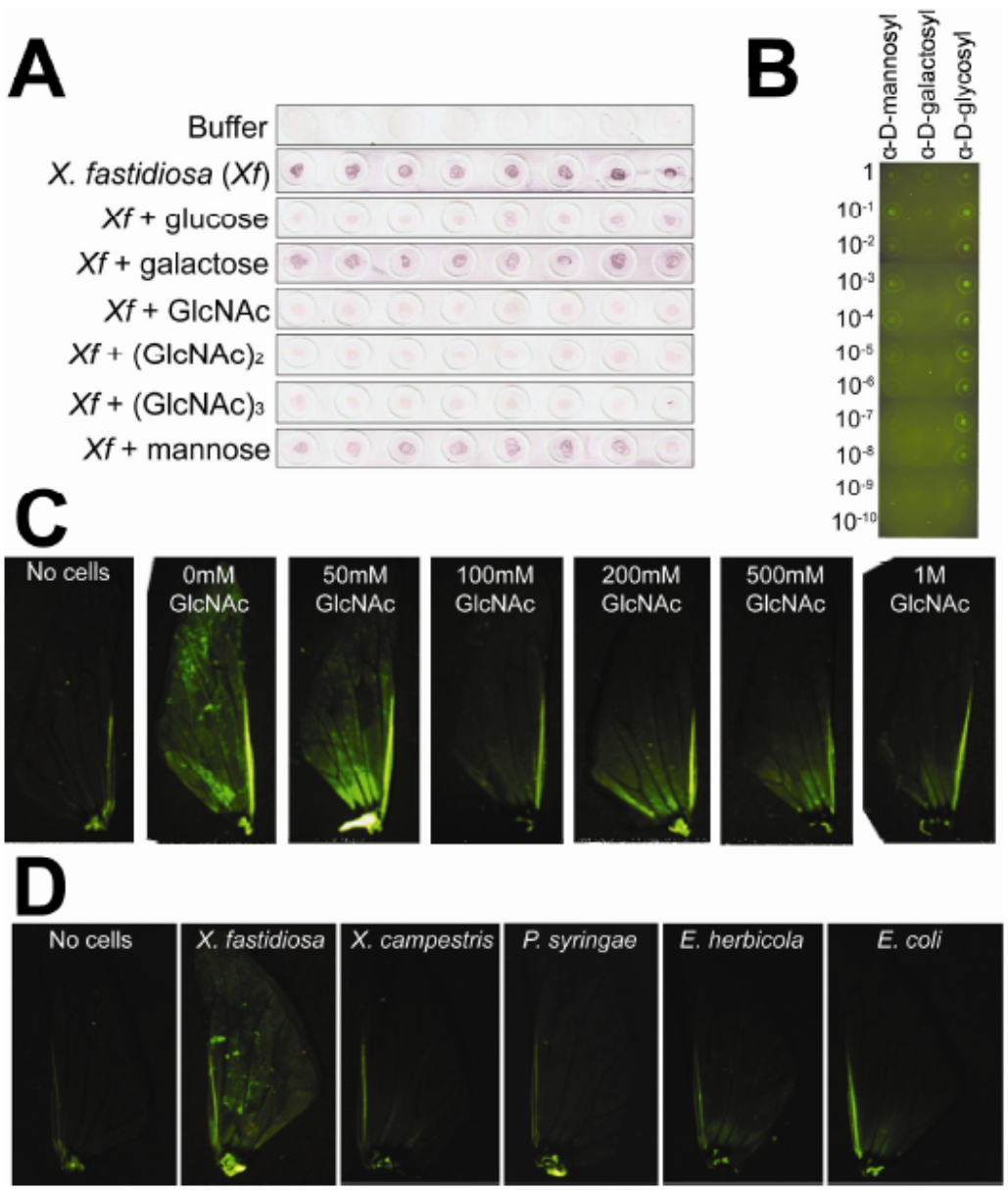


Figure 1. Carbohydrate-mediated inhibition of *Xf* cell attachment to surfaces. A) Carbohydrate inhibition of *Xf* attachment to leafhopper foregut extracts spotted on nitrocellulose membrane, indicating that cell surface adhesins can be saturated if incubated with certain molecules (GlcNac - *N*-acetylglucosamine).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

In order to compare our *in vitro* observations to *in vivo* cell adhesion to leafhoppers we used the hindwings of insect vectors to mimic the cuticular surface of the foregut canal that *Xf* colonizes. The entire exoskeleton of insects is generally assumed to have similar chemical composition, although details are lacking for this specific system. We used *N*-acetylglucosamine as competitor molecule in assays testing for GFP-labeled *Xf* cell attachment to hindwings. Attachment diminished as *N*-acetylglucosamine concentration increased in the dilution series (**Figure 1C**). These results indicate that *Xf* binding to polysaccharides *in vitro* is similar in its characteristics to its binding to the cuticle of leafhoppers. Lastly, in order to test the specificity of bacterial adhesion to leafhopper hindwings, we tested if other GFP-labeled bacteria, including the plant pathogens *Pseudomonas syringae*, *Xanthomonas campestris*, and *Erwinia herbicola*, and *Escherichia coli* attached to that surface (**Figure 1D**). Interestingly, only *Xf* cells attached to the wings. Thus, *Xf* cells have surface proteins with affinity to

polysaccharides on the surface of insects wings and glucosylated molecules, which can be saturated by *N*-acetylglucosamine and similar molecules.

Transmission of *hxfA*- and *hxfB*- mutants.
In previous reports, we presented biochemical results indicating that the hemagglutinin-like proteins (H*xf*A and H*xf*B) were associated to cell adhesion to insect surfaces and polysaccharides in vitro. Thus, we conducted two experiments to determine the role of *hxfA* and *hxfB* in *Xf* transmission by sharpshooters to plants. In the first experiment, we confined non-infected *G. atropunctata* on plants mechanically inoculated with the wild type, *hxfA*- and *hxfB*- cells, after which groups of two individuals were moved to healthy plants for four days as an inoculation access period. Transmission occurred in all treatments, with *hxfA*- and *hxfB*- being transmitted less than the wild type (70, 80 and 100%, respectively), albeit not with any statistical difference (X^2 test, $P = 0.1864$). In a second experiment we used individuals instead of pairs to more precisely estimate single insect transmission efficiency. With this more discriminating approach we found that *hxfA*- and *hxfB*- mutants were transmitted at lower rates than the wild type (36, 46 and 88%, respectively X^2 test, df = 1, $P < 0.001$). Because *Xf* transmission rates are correlated with bacterial population in plants, we quantified the infection level in plants used in these tests. Plants infected with *hxfA*- and *hxfB*- mutants used for the transmission tests had populations ~10-fold higher than the wild type (data not shown, results similar to Guilhabert and Kirkpatrick 2005), suggesting that *hxfA*- and *hxfB*- mutants were transmitted less than the wild type because of their impaired interactions with insects rather than because of lower populations in source plants.

We hypothesized that the reduced transmission rate of *hxfA*- and *hxfB*- mutants was due to limited colonization of vectors early in biofilm formation. In order to test this hypothesis we conducted another experiment and quantified the number of *Xf* cells in the head of vectors over time after a 12-hour pathogen acquisition access period. Overall, 80% of insects that fed on grapevines infected with the wild type were positive for *Xf*, whereas only 38% and 42% of those fed on *hxfA*- and *hxfB*- mutants were infected, respectively (X^2 test, df = 2, $P < 0.001$). We quantified the number of cells of these strains within vectors (positive samples only). There were significant effects of strain ($F_{2,54}$=23.229, P<0.0001), time ($F_{1,54}$=803.341, P<0.0001), and an strain by time interaction ($F_{2,54}$=5.362, P=0.0075). Populations of the two mutants soon after leafhopper access to infected plants were similar to each other but statistically different from the wild-type (**Figure 2B**). Twelve hours after acquisition we found insects fed on the wild type averaged 415 detectable cells, whereas average of 96 and 120 cells were detected in leafhoppers fed on *hxfA*- and *hxfB*- plants respectively. However, after 96 hours the bacterial populations of all three strains were similar to each other (**Figure 2B**). It was interesting to find that the slopes for the 2 mutants were similar (**Figure 2B**), suggesting that *hxfA* and *hxfB* may have redundant roles in relation to vector transmission and that, importantly, the knockouts were impaired in early attachment to insects, but after they attached, their patterns of foregut colonization (i.e. population growth) were similar to the wild type (slope of regressions). Testing of a *hxfA*-/*hxfB*- double mutant is necessary to determine if these proteins have redundant roles on cell attachment to vectors as our data suggest. However until recently, there were no protocols available for complementation studies with *Xf*, or to generate double mutants, prohibiting this test here (Reddy et al. 2007).

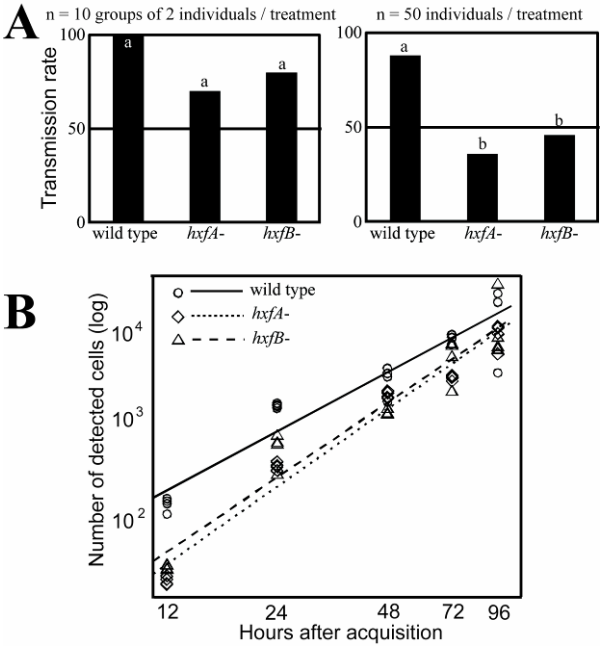
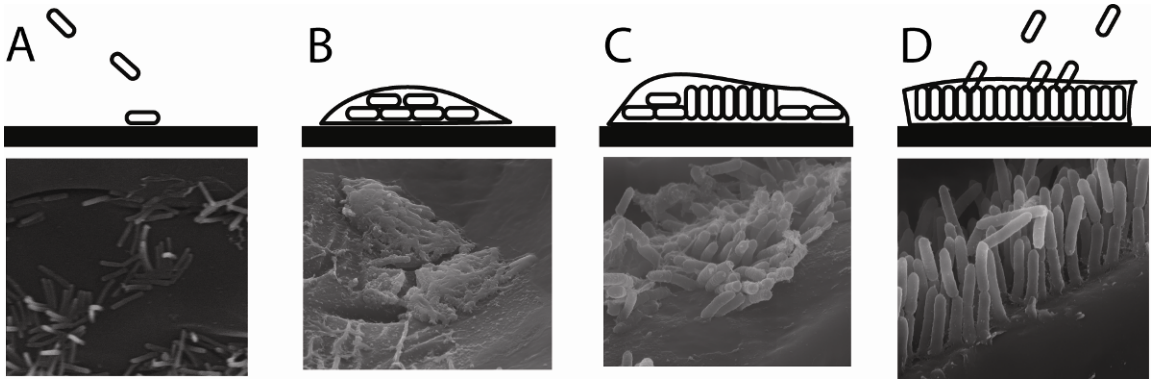


Figure 2. A) Transmission of *X. fastidiosa* by leafhopper vectors. Both experiments show that *hxfA*- and *hxfB*- were transmitted less often than the wild type, but results from larger experiment using individuals instead of groups were statistically significant. Different letters on bars indicate statistically difference ($P<0.05$). **B**) 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.

CONCLUSIONS

We propose that *Xf* colonization in vectors is similar to the formation of biofilms on surfaces. Scanning electron microscopy observations we have made support this hypothesis (Almeida and Purcell 2006). We hypothesize that cells initially adhere laterally to the foregut cuticle via carbohydrate-binding proteins, such as H*xf*A and H*xf*B (**Figure below A and B**). As these proteins are assumed to occur throughout cells, adhering laterally increases the cell surface area in contact with the substrate and streamline the bacteria to the flow of xylem sap ingested by the insect vector. After initial adhesion, cells may produce large quantities of EPS that can result in the concentration of resources and DSF in microcolonies. As the colony size increases, cells at the center of the biofilm became polarly attached (step C, below), potentially through polar short type I pili, increasing surface area for nutrient absorption. Lastly, a typical mature *Xf* biofilm within vectors is formed, with all cells polarly attached (step D). At this stage, newly divided cells are not anchored on the cuticle of insects and may be occasionally detached from vectors and inoculated into plants. This hypothesis may be useful to guide future studies on this system by providing testable questions, as up until know no data on these interactions, with the exception of microscopy observations, were available.



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